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Functional Analyses of Two Arabidopsis Apyrases

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Functional Analyses of Two Arabidopsis Apyrases

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Dedication

This dissertation is dedicated to the memory of my grandmother—
Xuehan Qiu

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Functional Analyses of Two Arabidopsis Apyrases

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ATP can serve as a signal molecule in the extracellular space to regulate biological processes and physiological effects in plant and animal cells. In mammalian cells, the level of extracellular ATP (eATP) is regulated by ectoapyrases, which can hydrolyze extracellular ATP to ADP and ADP to AMP. In this dissertation, I describe the important role of two Arabidopsis apyrases in the regulation of plant growth.

Seven apyrases have been identified in *Arabidopsis thaliana*. The genes for two of these seven apyrases, *APY1* and *APY2*, which have high sequence similarity, were cloned and characterized previously. The function of *APY1* and *APY2* was analyzed by T-DNA insertional mutant lines. The double knockout (DKO) apyrase pollen displayed a complete block of pollen germination, which implicated this step as the cause of the lethality of apyrase double knockout mutants. The vast majority of the mutant pollen grains were identical to wild-type in their nuclear state, and were viable as assayed by metabolic activity and plasma membrane integrity. Pollen tube elongation was inhibited

by suppression of apyrase activity using anti-apyrase antibodies or by chemical inhibitors of apyrases.

Etiolated hypocotyls overexpressing *APY1* (with expression driven by a constitutive Cauliflower Mosaic Virus (CaMV) *35S* promoter) exhibited faster growth rates compared to wild-type plants. Because of the lethality of *apy1apy2* double mutants, RNA interference (RNAi) was performed as an alternative approach to post-transcriptionally silence the expression of apyrases. The suppression of apyrases in the RNAi lines resulted in a dwarf phenotype in overall vegetative growth and dramatically reduced growth in primary root and etiolated hypocotyls. In addition, the RNAi mutant plants lacked a well-defined meristematic zone and had a greatly reduced elongation zone in the primary root. Previously, promoter-GUS fusions showed that high expression of apyrase was associated with areas of rapid growth and regions with high auxin levels. Abnormal auxin accumulation was found in the proximal regions of the primary roots of RNAi mutant plants, which demonstrated that the absence of apyrase results in disrupted auxin distribution. Other phenotypes in RNAi mutant plants, such as less lateral root formation and more adventitious roots, could also be associated with abnormal auxin distribution. The investigation of the subcellular localization of apyrases showed that some fraction of apyrase was localized on cell periphery. These results suggest that the expression of APY1 and APY2 is essential for plant growth. They favor the hypothesis that Arabidopsis apyrases, like their homologs in animals, control the levels of ATP in the extracelluar space, and this control allows them to act as key regulators in growth.

Table of Contents

List of Figures

CHAPTER 1: INTRODUCTION

Apyrases (NTPDases) are enzymes that can catalyze the hydrolysis of nucleoside tri- and/or diphosphates, but not nucleoside monophosphates or non-nucleoside phosphates. They were first described by Meyerhof in 1945 (Meyerhof 1945) and are commonly found in both prokaryotic cells and a wide variety of eukaryotic cells. In animal systems, apyrases are well known to be involved in multiple systems and to carry out different functions. Our knowledge of the functions of apyrases in plants, especially in Arabidopsis, is still quite limited and needs further investigation.

CHARACTERISTICS AND CLASSIFICATIONS OF APYRASES

Apyrases belong to a special type of ATPase, the E-type ATPases. E-type ATPases are distinguishable from other types of ATPases by sharing some common characteristics, which usually include activation by either Ca^{2+} or Mg^{2+}, relative insensitivity to inhibitors of F-type, P-type, and V-type ATPases, and ability to hydrolyze both nucleoside triphosphates and nucleoside diphosphates.

The enzyme activities of apyrases are divalent cation dependent. The divalent cations that can activate apyrases include Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} (Guranowski et al. 1991). More recently, a completely new type of apyrase showed only dependence upon Ca^{2+} ions but no other divalent cations. This was identified from the bed bug *Cimex lectulariu* (Valenzuela et al. 1998). Unlike most ATPases, which have a preference to hydrolyze ATP, apyrases show relatively low substrate specificities. Apyrases

metabolize not only ATP or ADP but also a broad range of purine and pyrimidine 5'-trinucleotides. Apyrases also exhibit insensitivity toward inhibitors of P-type, F-type, and V-type ATPases and most inhibitors of alkaline and acid phosphatases (Zimmermann 2001, Steinbrunner et al. 2003).

Apyrases can be classified into two major forms, a soluble form, which is isolated from the cytosol of plant and insect cells, and a membrane-bound form. The membrane-bound apyrases can be divided into two groups based on the location of the catalytic site. Ectoapyrases have their catalytic site facing out towards extracellular space, while the endoapyrases work intracellularly by having their catalytic site within the borders of the plasma membrane. In animals, most apyrases are extracellular apyrases, and they participate in regulation of 5'-nucleotidase and adenylate cyclase activity, as well as blood platelet aggregation (Komoszynski and Wojtczak 1996).

The sequence alignment of apyrase proteins demonstrated five highly conserved regions called apyrase conserved regions (ACRs). ACRs 1 through 4 were first defined in potato tubers (Handa and Guidotti 1996) and ACR 5 was identified by Vasconcelos et al. (1996) in animals. The sequence analysis of ACR1 and ACR4 shows they have high similarity to β- and γ-phosphate binding motifs that belong to the actin-hsp 70-hexokinase family (Handa and Guidotti 1996). A recent study of human nucleoside triphosphate diphosphohydrolase 3 (eNTPDase-3) revealed that ACRs are essential for controlling the biological activities of apyrases, such as catalytic activities and substrate specificity. Altered and diminished substrate specificity and reduced enzymatic activities were observed in mutants with site-directed mutagenesis in several conserved residues within ACRs (Kirley et al. 2001, Yang et al. 2001).

EXTRACELLULAR ATP (EATP)—SOURCE, RECEPTORS, BIOLOGICAL EFFECTS, AND METABOLISM

Adenosine triphosphate (ATP) is commonly recognized as a metabolic energy source in cells. It is also known as a signaling molecule that can modulate a variety of biological responses through extracellular signaling (Burnstock and Knight, 2004). ATP is first produced in mitochondria by ATP synthases, via the process of oxidative phosphorylation and later transported to the cytosol. Although the mechanism for releasing ATP to the extracellular milieu is still unknown, various mechanisms have been described in different studies. Some evidence suggests that ATP might be symported along with other substrates through an ATP channel by some protein modulators, such as multidrug resistance proteins (MDR) and cystic fibrosis transmembrane conductance regulator (CFTR) (Roman et al., 2001). In normal conditions, the extracellular ATP concentration is much lower (less than 0.1 μM) than the intracellular ATP concentration, which is present at 1 mM to 10 mM (Coade and Pearson 1989, Zimmermann 1994, 1998). Some studies indicate that ATP might flow out of cells following this steep intracellular to extracellular gradient through ATP permeable channels (Braunstein et al. 2001, Jackson and Strange 1995). ATP also can be packaged into secretory vesicles or granules and actively released out of the plasma membrane via regulated exocytosis (Burnstock 1995, Gordon 1986,). In addition, large amounts of ATP can be passively released by physically injured cells at a wound site (Dubyak and El-Moatassim 1993, Song et al. 2006).

For ATP to act as an extracellular signal, there must be some receptor to perceive eATP levels, and these receptors have been identified in animals. Purinergic receptors are proteins that are localized to the surface of the plasma membrane and bind to the nucleotides in the extracellular space. There are two groups of purinergic receptors in animals—P1 receptors and P2 receptors.

P1 receptors are activated primarily by adenosine, whereas P2 receptors bind to ATP, ADP, UTP and UDP as agonists (Ralevic and Burnstock 1998, Dubyak and El-Moatassim 1993). The P2 family is further divided into two subgroups--purinergic receptor type X (P2X) and purinergic receptor type Y (P2Y) based on pharmacological classification (Ralevic and Burnstock 1998, Di Virgilio et al. 2001).

The protein structure of P2Y receptors consists of seven transmembrane-spanning segments with the N-terminal located in the extracellular space and the C-terminal inside the cell. P2Y receptors are G-protein coupled and are activated by adenine and uridine nucleotides. The response mediated by P2Y receptors is to induce the activation of phospholipase C, the production of inositol triphosphate (IP_3), and an increase in cytosolic Ca^{2+} concentration (Fredholm et al. 1994).

P2X receptors are ligand-gated ion channels activated by extracellular ATP. So far, seven P2X receptors ($P2X_1$-$P2X_7$) have been identified in this family (Burnstock 2002). P2X receptors contain two transmembrane subunits, with both C- and N-terminals localized inside the cell, and have an extracellular loop to bind ATP. In response to ATP, P2X receptors increase the permeability of the cell membrane and allow cations (Ca^{2+}, Na^{+}, K^{+}) to cross the membrane from the extracellular space. This

leads to a depolarized cell membrane resulting in the opening of a voltage-gated Ca^{2+} channel that elevates the intracellular Ca^{2+} concentration (Buell et al. 1996).

In animals, extracellular nucleotides elicit a wide variety of physiological effects through P2 receptors. They are involved in inflammatory processes, sensation of pain, regulation of epithelial cell functions, mediation of the cardiac rhythm and contractions, the process of apoptosis, and platelet aggregation (Volonté et al. 2006, Gordon 1986, Schwiebert 2003), to name just a few.

Is ATP a signal molecule in plants? This question has been increasingly raised by a number of publications in recent years. In the early 70's, Jaffe (1973) reported that exogenously applied ATP could promote the closure of the Venus'-Flytrap. Studies on lily pollen revealed that externally applied ATP could enhance mitotic division in pollen tubes (Kamizyo and Tanaka 1982). External ATP is also involved in mediating the stomatal opening in *Commelina communis L.* (Nejidat et al. 1983). In explaining these data, authors proposed that the external ATP was functioning as an energy source. More recently, Lew and Dearnaley (2000) reported that 1 mM ATP, ADP, and GTP can induce membrane depolarization up to 100 mV in root hairs. Detection of increased extracellular ATP was found in Arabidopsis plants overexpressing a multidrug resistance gene *MDR1* (Thomas et al. 2000). Previous studies of the *MDR1* gene in animal cells showed it may function as an ATP channel to export ATP into the extracellular space (Abraham et al. 1993), and a similar mechanism for ATP release may be present in plants. Studies on both Arabidopsis roots and whole seedlings showed that exogenous ATP could significantly increase the cytosolic calcium concentration in plants (Demidchik et al. 2003, Jeter et al. 2004).

Once a signal transduction pathway is activated, it needs to be terminated properly for the correct response to occur. The hydrolysis of extracellular ATP can be done by ecto-enzymes expressed on the surface of the cell membrane, such as ectoapyrases, ecto-nucleotide pyrophosphatases, alkaline phosphatases, and ecto-5'-nucleotidases (Zimmerman 1996). The process is initiated by ectoapyrases and terminated by ecto-5'-nucleotidases. Thus by degrading ATP into its metabolites, the function of ectoapyrases might be to modulate nucleotide mediated signal responses.

APYRASES IN MAMMALIAN CELLS

Apyrases are commonly called E-NTPDase (ecto-nucleotide triphosphate diphosphohydrolase) in mammalian cells. Eight mammalian apyrases have been cloned and characterized—NTPDase 1-8 (Bigonnesse et al. 2004). Most members of the E-NTPDase family are membrane-bound proteins, either on the plasma membrane or Golgi membranes, although NTPDase 5 is a secreted soluble enzyme (Shi et al. 2001, Mulero et al. 1999). NTPDases 1-3 can exist as dimers, trimers, or tetramers, but NTPDase 5 only exists as a monomer (Zimmerman 2000). NTPDases differ in preferences of hydrolyzing ATP and ADP. NTPDase 1 can degrade both ATP and ADP equally. NTPDase 2 hydrolyzes ATP far more efficiently than ADP (about 30-fold). NTPDase 3 and 8 show slightly higher activities of hydrolyzing ATP over ADP (Robinson 2006).

The regulation of P2 receptors is strongly influenced by the biochemical characteristics of individual NTPDases. NTPDases 1, 2, 3, and 8 can hydrolyze UTP and ATP and could be involved in terminating the stimulatory effects of these nucleotides on P2X and P2Y. NTPDase1 can degrade both ATP and ADP to generate AMP. The

production of AMP in turn can promote the activation of adenosine receptors after the conversion of AMP to adenosine. NTPDase 2, 3, and 8 can cause the accumulation of ADP and thus provide the agonist for ADP receptors, such as $P2X_1$, $P2Y_1$, and $P2Y_{12}$ (Kukulski et al. 2005, Remijn et al. 2002).

NTPDase 1, originally known as CD39, is the prototypic member of the ectoapyrases. It is a 70-100 kDa integral membrane protein and was originally found as an activation marker on the surface of activated lymphoid and endothelial cells (Rowe et al. 1982, Malizaewski et al. 1994). CD39 was first identified as an ectoapyrase by Wang and Guidotti in 1996 (Wang and Guidotti 1996). The CD39 protein is composed of a large extracellular region, which contains five apyrase conserved regions (ACRs) and two transmembrane domains near the amino- and carboxy-termini (Malizaewski et al. 1994). The external part of CD39 may be critical for its enzymatic activities (Handa and Guidotti 1996, Zimmermann 2001). Some detergent solutions can affect the enzymatic activity of CD39 by affecting the oligomeric structure of the two transmembrane domains, whereas the detergent-solubilized version of CD39 is not affected (Wang et al. 1998). CD39 is originally synthesized in the endoplasmic reticulum (ER) and transported to the plasma membrane via secretory vesicles. The active site of CD39 faces the exterior of the cell, and the enzyme does not exhibit activity until it arrives at the cell surface (Zhong et al. 2001).

N-glycosylation is an important posttranslational modification that can influence the localization of a protein. CD39 is a highly glycosylated protein with six potential N-linked glycosylation sites. A recent study showed that eliminating N-glycosylation can affect the plasma membrane localization of CD39, and the activated form of CD39 is

more glycosylated than the intracellular form, which is the inactivated form (Zhong et al. 2001).

More recently, scientists have focused on the function of CD39 as a thromboregulator, and its potential role in therapeutic application in thrombotic disorders (Qawi and Robson 2000). Abnormal platelet aggregation can cause unregulated homeostasis and further the formation of thrombus. In endothelial cells, one biochemical mechanism to control platelet activity is through endothelial ectoapyrase--CD39. CD39 is highly expressed on the endothelial cell surface and is substrate activated. It can convert ADP, which triggers the platelet aggregation, to AMP and thus inhibit thrombosis (Marcus et al. 2001).

In the immune system, CD39 was first found in B lymphocyte cells, but it is also expressed in many other cell types, such as natural killer cells, monocytes, dendritic cells and activated T cells (Dwyer et al. 2007). CD39 and other ectoenzymes can modulate leukocyte migration during immune responses by regulating the adenosine levels in the cell (Salmi and Jalkanen 2005). Experiments with *cd39* null mice showed increased extracellular ATP levels and defects of antigen-presenting function and formation in dendritic cells, and impaired T cell responses to haptens (Mizumoto et al. 2002, Dwyer et al. 2007).

ATP is recognized as a fast neurotransmitter in the nervous system and has been found in both central and peripheral nerves. ATP can induce physiological effects by activating the P2 receptor. This P2 receptor signaling will be terminated by an ecto-nucleotidase cascade. When ATP is released in the synaptic space, it can cause the depolarization of a postsynaptic membrane and contribute to signal transmission to the

adjacent neuron or neuron-muscular junction (Komoszynski and Wojtczak 1996). The expression of CD39 has been detected ubiquitously in the nervous system. The major function of CD39 is to hydrolyze ATP to AMP, which has lower affinity for the receptor, thereby reducing receptor activation and subsequently inhibiting neurotransmission (Komoszynski and Wojtczak 1996).

ATP can also modulate the release of other neurotransmitters, such as norepinephrine. Norepinephrine as a neurotransmitter can be released as cotransmitters with ATP. Recently Machida et al. (2005) reported that CD39 could act as a regulator to negatively mediate norepinephrine exocytosis by inactivating ATP in nervous system.

In animals, the major function of apyrase includes terminating and modulating the signal effects triggered by extracellular nucleotides, producing AMP, which is a source of adenosine. AMP activates the adenosine-induced signal transduction chain, controlling the amount of extracellular nucleotides as the agonist to the P2 receptors, thereby canceling the effects caused by ADP during platelet aggregation, and releasing the nervous system receptor. Thus apyrase plays an important role in modulating the biological effects induced by ATP or ADP in different tissues and systems.

APYRASES IN YEAST

In yeast, two apyrases have been identified so far— GDA1 and YND1 (Abeijon et al. 1993, Gao et al. 1999). Both apyrases exist as endo-apyrases and are integral membrane proteins localized to the Golgi membrane. GDA1 can function as a GDPase and UDPase with higher activity to hydrolyze GDP over UDP, but no activities with other nucleotides, whereas YND1 has wide substrate specificity and can hydrolyze

nucleoside tri- and diphosphates. Sugar transportation in Golgi is important for post-translational modification of proteins, and the sugars enter the Golgi by an antiport system. GMP exits the Golgi through the antiport system and is accompanied by the influx of GTP-sugar (Abeijon et al. 1993, Gao et al. 1999). Zhong et al. (2000) showed that Vma13p, which is an activator subunit of the V-ATPase complex, regulates the activity of YND1. It represses the activity of YND1 by binding to the cytoplasmic domain of YND1.

The functions of GDA1 and YND1 partially overlap. Yeast without both of these genes exhibit the loss of *N*- and *O*-linked glycosylation and defects in cell-wall formation and cell growth (Gao et al. 1999).

APYRASES IN INSECTS

Platelet aggregation is one of the haemostatic responses after vascular injury, and in response to damage the ADP concentration is significantly increased in the extracelluar space. To obtain the blood, blood-feeding insects have developed some adaptive compounds to inhibit the factors released from haemostatic responses. Soluble apyrases are found ubiquitously in saliva of blood-sucking bugs. These endoapyrases act as a platelet-inhibiting agent to reduce the concentration of ADP in damaged tissue and prevents platelet aggregation (Valenzuela et al. 1996, Cheeseman 1998, Gayle et al. 1998, Mans et al. 2000).

APYRASES IN PLANTS

At least 18 apyrases have been found in a variety of plant species, including legumes (4) (Cohn et al. 2001, Etzler et al. 1999, Hsieh et al. 1996), Arabidopsis (7) (Steinebrunner et al. 2000), potato (1) (Handa and Guidotti 1996), and *Mimosa pudica* (6) (Ghosh et al. 1998). Apyrase from potato tuber was the first apyrase that was characterized over 60 years ago (Meyerhof 1945). Many isoforms of potato apryases have been identified, but only one soluble apyrase has been purified and cloned (Honda and Guidotti 1996). The postulated role of apyrase in potato tuber is in regulating starch synthesis and metabolism, which is modulated by the level of ATP, ADP, or phosphate (Honda and Guidotti 1996).

The function of plant apyrases has been intensively studied in leguminous plants, such as *Dolichos biflorus*, soybean (*Glycine soja*), *Medicago truncatula*, *Lotus japonicus* and pea (*Pisum sativum*). Three apyrases—PsAPY1, PsAPY2, and psNTP9 were identified in pea. psNTP9 was originally purified from etiolated pea plumule nuclei and reported to respond to light stimuli. The activity of psNTP9 can be up-regulated by calmodulin and casein kinases II (Chen and Roux 1986, Chen et al. 1987, Hsieh et al. 1996, Hsieh et al. 2000). Thomas et al. (1999) reported that in light-grown tissue a significant fraction of psNTP9 co-purified with purified plasma membranes and was involved in mediating phosphate uptake from the extracellular space. Overexpressing this apyrase in wild type Arabidopsis improved the xenobiotic resistance of the transgenic plants, suggesting that apyrase might play a role in toxin resistance (Thomas et al. 2000). Studies of other laboratories showed that some of the apyrase isolated from pea stems is

associated with the cytoskeleton and suggested that the protein might be involved in cytoskeleton-related signal transduction and transportation (Shibata et al. 1999).

In some legumes, apyrases appear to be associated with interactions between plants and microorganisms. *Mycosphaerella pinodes*, a pathogenic fungus, can either stimulate or inhibit the ATPase activity of pea cell wall-bound apyrases by secreting a glycoprotein elicitor or a mucin-type glycopeptide suppressor (Kiba et al. 1995). Thus apyrases may respond to the extracellular signals that regulate the interaction of a plant with its environment (Takahashi et al. 2006).

Another response to the environment mediated by apyrases is the Rhizobium-legume symbiosis. This is the process in which nitrogen-fixing rhozobial bacteria infect a leguminous species and establish nodules inside of the host roots. Nod factors are modified lipo-chitooligosaccharides secreted by rhizobia and act as the signals that trigger the formation of root nodules with members of the legume family.

A lectin isolated from the roots of the legume, *Dolichos biflorus*, had apyrase activity, and sequence analysis of this lectin-nucleotide phosphohydrolase (LNP) showed four apyrase-conserved regions (Etzler et al. 1999). The results of immunofluorescence assays revealed that Db-LNP is localized on the epidermal cell surface of young roots and is also abundant on the surface of root hairs, where the infection of rhzobial bacteria takes place. Db-LNP had high affinity to bind with Nod-factor and its ATPase activity was stimulated after the binding. When anti-LNP serum was applied to it, the root showed a reduced ability to form nodule and root hair deformation, which is one of the early symptoms before nodule formation (Etzler et al. 1999). Further investigation showed that Db-LNP is a peripheral membrane protein on the root surface and was also localized

to the root pericycle. When the roots were treated with symbiotic rhizobia there was a redistribution of Db-LNP from root surface to the root tip. Controls indicated that this response is not due to a pathogen defense response (Kalsi and Etzler 2000). Etzler's group concluded that Db-LNP can be a good candidate as a receptor to activate the downstream events in the early stage of the rhizobium-legume symbiosis.

Two sobean apyrases—GS50 and GS52 were characterized by Day et al. (2000). The study of these two apyrases showed that GS50 is an endo-apyrase localized in Golgi whereas GS52 may function as an ectoapyrase localized to the plasma membrane. Just as anti-LNP serum can inhibit nodule formation, similar results were found by using antibody raised against GS52. Phylogenetic analysis revealed that GS52 is closely related to Db-LNP and pea apyrase (Day et al. 2000). McAlvin and Stacey (2005) reported that enhanced nodulation, increased root hair infection, and expanded infection areas were found in transgenic *Lotus japonicus* overexpressing GS52.

The distribution of GS50 and GS52 was slightly different. GS50 but not GS52 was found to be highly expressed in the stem of 5-day-old seedlings. Even though both proteins are expressed in roots, GS52 showed a much higher expression than GS50. Surprisingly high expression of both proteins was detected in flowers. Their different distribution suggests these two soybean apyrases might be involved in different cellular processes in the plant (Day et al. 2000).

Six apyrase genes were identified in *Medicago truncatula*, including *MtAPY1;1, MtAPY1;2, MtAPY1;3, MtAPY1;4, MtAPY1;5, and MtAPY2)* (Cohn et al. 2001, Navarro-Gochicoa et al. 2003). MtAPY1;1 to MtAPY1;5 are proteins that belong to the legume-specific apyrase family, whereas MtAPY2 is more similar to Arabidopsis apyrases.

Transiently increased mRNA levels of *MtAPY1;1* and *MtAPY1;4* were observed after 3 and 6 hours inoculation with rhizobia, suggesting that these two apyrases may function as early nodulin genes (Cohn et al. 2001).

Arguing against this conclusion, Navarro-Gochicoa et al. (2003) reported that similar results could be obtained using aeroponic chambers but not growth pouches. The mRNA levels of *MtAPY1* genes were examined in different symbiotic mutant lines. The results showed that the increased mRNA levels might not be the result of the response to nodule formation. Navarro-Gochicoa et al. (2003) discussed several possible alternative explanations, but noted that more evidence is needed.

Based on sequence analysis, seven apyrase genes were identified in the *Arabidopsis thaliana* genome. *Atapy3, 4,* and *5* were mapped in tandem on chromosome 1, and *Atapy6, 1, 7,* and *2* were on chromosome 2, 3, 4, and 5, respectively. The seven Arabidopsis apyrases can be divided into three groups based on amino acid sequence similarity. AtAPY1 and 2 share over 87% protein sequence identity, and these two apyrases are more similar to MtAPY2 and PsAPY2 (Cohn et al. 2001, Steinebrunner et al. 2000). The second group consists of AtAPY3, 4, 5, and 6. AtAPY7, which has low similarity to any other apyrase, belongs to the last group. The different substrate specificities of pea and Arabidopsis apyrases showed a trend of ATP>CTP>GTP>UTP (Chen and Roux 1986).

AtApy1 (*APY1*) and *AtApy2* (*APY2*) were originally cloned by Steinebrunner et al. (2000). Sequence analysis revealed that APY1 contains a calmodulin-binding motif, and further investigation showed that the APY1 protein could bind to calmodulin in the presence of Ca^{2+} (Steinebrunner et al. 2000). Promoter-GUS staining showed that

APY1 and *APY2* have similar expression in some tissues and differentially expressed in other tissues. High-levels of expression of both genes can be obtained in mature embryo, primary root, lateral root, root-hypocotyl junction, etiolated hypocotyl, mature pollen grains, the surface of stigma, pollen tubes, and abscission zone. Strong AP2:GUS activity has also been detected in the zone of elongation in root, but not in AP1:GUS transgenic lines.

The expression patterns of theses two apryases are also regulated by light. GUS staining can be detected both in the hypocotyls and roots of etiolated seedlings, whereas in the light only roots showed GUS activity (Wu et al. 2007). When using AP1:GUS and AP2:GUS to transform the phytochrome mutants *phyA*, *phyB*, and *phyA/B*, results showed the detection of GUS activity in light grown hypocotyls. This suggested that phyA and phyB might act as upstream regulators of the expression of *APY1* and *APY2* in light.

In experiments carried out by Dr. M. Salmi, the results of the promoter:GUS assays carried out in etiolated hypocotyls were confirmed by RT-PCR assays, which showed that transcript levels for *APY1* and *APY2* dropped by more than 80% within 15 min after a 4 min red-light irradiation (Wu et al. 2007). Moreover, Western analyses carried out by T. Butterfield showed that immunodetectable apyrase decreased by more than 80% within 7 min after the start of a 4 min red-light irradiation (Wu et al. 2007).

Changes of apyrase expression were detected at the wound site, as judged by promoter:GUS assays. The transcript level of *APY1* gene can be upregulated by wounding and reaches a peak at 1 hour post wound, but *APY2* transcript levels do not respond to wounding (Y. Sun, Ph.D. Thesis). Large amounts of ATP can be released

into the ECM of plant cells upon wounding and can induce the accumulation of superoxides, which act as a mediator of wound response in plants. Overexpression of APY2 reduced the superoxide production normally induced by wounds in Arabidopsis leaves, most likely by reducing the [eATP] released into the intercellular spaces of wound sites. This indicates that apyrases may play a role in the signaling responses induced by the extracellular ATP released at a wound site (Song et al. 2006)

To study the function of these two Arabidopsis apyrases, T-DNA insertional knockout mutants were generated from a T-DNA pool. *apy1* and *apy2* single null mutants did not show any phenotype when evaluated under different conditions. However, knocking out both apyrases is lethal (Steinbrunner et al. 2003).

In this dissertation, I tested hypotheses about the lethality of double-knockout plants. I found that double-knockout pollen grains fail to germinate, which accounts for the lethality of double-knockout plants. I also carried out further apyrase suppression studies by an inducible RNAi system and showed that the reduction of both apyrases can lead to dramatically reduced root elongation, decreased etiolated hypocotyl growth, and a dwarf phenotype. Additionally, I tested the endogenous auxin distribution in roots of RNAi lines, and the results revealed that the accumulation of high levels of auxin in tip regions of roots might be the cause of the root phenotype. Taken together, our results indicated that APY1 and APY2 play crucial roles in regulating pollen germination and plant growth, probably by regulating the concentrations of extracellular ATP in Arabidopsis.

CHAPTER 2: APYRASES PLAY A CRUCIAL ROLE IN POLLEN GERMINATION AND TUBE ELONGATION

INTRODUCTION

Sexual reproduction of flowering plants is the process whereby sperm are delivered to egg cells by a pollen tube. The male gametes and the pollen grains that contain them develop and mature in anthers, which are known as the male sexual reproduction organ. In Arabidopsis, four microspores are produced after meiotic division followed by two mitotic divisions. The first mitotic division, also called pollen mitosis I (PM I), is an asymmetric division and gives rise to two cells, one large vegetative cell and one small generative cell. After the first mitotic division only the generative cell undergoes another division and forms two sperm cells. This process is called pollen mitosis II (PM II).

After two mitotic divisions, dehydrated pollen grains are ready for pollination. Pollination takes place when mature pollen is shed from the anther and lands on the surface of the stigma. It involves cell-cell recognition and inter- and intracellular signaling between male gametes and the pistil. However, understanding of this process at the molecular level is still incomplete. Once a pollen grain is recognized and accepted, it starts absorbing water and nutrients and begins to germinate. Once pollen germinates, the pollen tube elongates at a remarkable rate to penetrate the style and deliver the sperm to the final destination--the ovary. Pollen tubes are known to display a tip growth requiring the establishment of polarity and the incorporation of new

membrane and wall materials by secretory vesicles at the apical tip. This requires a functional actin cytoskeleton.

A mutation described by Schiefelbein et al. (1993) had defective growth in both pollen tube elongation and root hair growth. Thus genes controlling tip growth may be similar even in different cell types. Ca^{2+}-mediated signaling is important for pollen germination and tube elongation. The establishment of a cytoplasmic calcium gradient caused by the influx of calcium is crucial for the tip growth (Heslop-Harrison and Heslop-Harrison 1992a, 1992b; Franklin-Tong 1999). Some studies have shown that the oscillating growth rate of pollen tubes might be correlated with changes in cytosolic free Ca^{2+} concentration (Pierson et al. 1996). Ca^{2+} can also function as a secondary messenger in signaling pathways of pollen germination and tube growth.

Calmodulin (CaM) is a protein that can interact with and bind to Ca^{2+} in signal transduction pathways. CaM can associate with myosin during pollen germination, but this association is reduced at high Ca^{2+} concentrations (Franklin-Tong 1999). Rop/Roc GTPases are members of Rho-GTPase family and are known to play a role in regulating the pollen tip growth by controlling the Ca^{2+} influx and gradient (Li et al. 1999, Franklin-Tong 1999). Other signaling components that are also involved in regulating pollen tube growth include calcium-dependent protein kinase, other protein kinases, phosphoinositides, and actin-binding proteins (Franklin-Tong 1999).

Previous studies from Dr. Roux's lab identified two apyrases (*APY1* and *APY2*) that have high-level expression in mature pollen grains and pollen tubes of Arabidopsis during post-pollination stage (Steinbrunner et al. 2003). These two apyrases share 87% identity at the amino acid level (Steinbrunner et al. 2000). The *apy1* and *apy2* single

knockout plants were obtained from a T-DNA insertional pool and tested for phenotypes under different conditions. However, neither *apy1* nor *apy2* single knockouts exhibit obviously different phenotypes compared to wild-type controls. Attempts to generate double knockout plants failed, suggesting that knocking out both *APY1* and *APY2* may be lethal.

If the double knockout plants are lethal, they may be embryo lethal, male sterile, or both. In order to distinguish between these possibilities, seed development was tested by counting both the number of seeds in siliques and the germination ratio of plants heterozygous for both apyrases (*APY1apy1*; *APY2apy2*). These plants were obtained by crossing homozygous *apy1* single knockout plants (*apy1apy1*; *APY2APY2*) with *apy2* single knockout plants (*APY1APY1*; *apy2apy2*). However, both the number of seeds and the germination rates in these plants were normal compared with wild-type controls (data not shown). These results indicated there was no defect in seed development and suggested that the double knockout plants had altered pollen development or viability. Pollen germination and tube elongation are key steps during plant reproduction and require the expressions and regulation of a large number of genes (Park et al. 1998, Chen and McCormick 1996, Li et al. 1999, Franklin-Tong 1999). In this chapter I will discuss the critical role of two Arabidopsis apyrases in pollen germination and tube elongation.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana ecotype Wassilewskija (WS) was used as wild-type in pollen germination studies of knockout lines. The *qrt1-2* mutants were obtained from TAIR, stock number CS8846. Seeds were sown on soil directly and stored at 4 °C for 3 days before starting growth at 23 °C with 24 hr light. Genotypes of heterozygous knockout lines were identified by PCR.

Pollen germination assay

For pollen *in vitro* germination assays, flowers at stages 13 to 14 were picked from plants no later than two weeks after bolting. After 2 hours dehydration at room temperature, pollen grains were dipped on the pollen germination medium (0.01% boric acid, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, 18% Sucrose, and 0.5% agar, pH 7.0) (Li et al., 1999) on a regular microscope slide.

For the effect of apyrase inhibitor in pollen germination, pollen from wild-type flowers was germinated in liquid germination medium containing 0.03% $CaCl_2$, 0.01% H_3BO_3, 12% Sucrose, and 0.2% DMSO (Torres et al., 1995) in depression slides. Apyrase inhibitor NGXT1913 (Windsor et al., 2002) (Texagen Inc. Austin, TX) was added and mixed with the medium before pollen was applied. For both pollen germination and inhibitor assays, slides containing pollen germination medium and pollen grains were put into 150 mm petri dishes with wet filter paper to maintain high humidity. Pollen grains were incubated in a chamber at 27 °C with 16-24 hours.

Pollen viability and cytochemical analysis

Pollen viability assays were performed by incubating fresh pollen with fluorescein diacetate. The fluorescein diacetate solution was made by dissolving 0.2 mg fluorescein diacetate powder in 1 ml acetone and then adding the mixture to 10 ml of 10% sucrose solution drop by drop until the solution turned milky. Pollen grains were incubated with flurescein diacetate 5-10 minutes before viewing with a fluorescence microscope (Heslop-Harrison and Heslop-Harrison, 1970).

For pollen nuclei staining, 0.5 µg/ml 6-diamidino-2 phenylindole (DAPI) was dissolved in buffer solution (50 mM $NaPO_4$, 1 mM EDTA, and 0.1% Triton, pH 7.0). Pollen was dipped on pollen germination medium containing 0.5% agar (Howden et al. 1998). Then 200 µl of the DAPI solution was applied on top of the medium for 5–30 min and followed by two brief washes with the same buffer. Pollen was viewed by a Leica DM IRBE microscope under UV by epi-illumination.

Assay of pollen tube elongation by apyrase antibody and apyrase inhibitors

Wild-type pollen was germinated in pollen germination medium (1.6 mM boric acid, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, and 5 mM HEPES buffer in 18% sucrose, 1% agar, pH 7.0) in depression slides at 26 ºC for 4 hours in the dark. Only slides that achieved at least a 60% germination rate were used in the experiment. At least 20 pollen tubes were measured for each treatment to get a representative growth rate of the tubes in each slide.

Two different concentrations were tested both in pre-immune serum and protein-A purified immune serum (preimmune sera: 0.3μg/μL and 0.6 μg/μL; immune sera: 0.4 μg/μL and 0.8 μg/μL). For the effects of apyrase inhibitors on pollen tube growth

inhibitors NGXT 191 (7.7 μM) and NGXT 194 (7.9 μM) (Windsor et al., 2002) (Texagen Inc. Austin, TX) were used. Then 150 μL pollen germination solution containing serum or apyrase inhibitors were applied to the top of the germinated pollen. 0.1% Dimethylformamide was used as a control in the apyrase inhibitor assay. Pictures were taken at 1 min (Time 0) and 15 min (Time 1) after the solution application using a PixeLINK PL-662 microscopy camera. The growth rates were calculated (rate/h = (length of Time 1 – length of Time 0)/15 x 60).

Genetic crosses for tetrad analysis

For the tetrad analysis, *apy1* mutant plants and *apy2* mutant plants were crossed with *qrt1-2* (CS8846) (Preuss et al. 1994), respectively. The F1 generation plants were the heterozygotes of the two parents—(*APY1apy1; APY2APY2; QRTqrt*) and (*APY1APY1; APY2apy2; QRTqrt*). The F2 generation plants were obtained by self-crossing the F1 plants, which have both single knockout apyrase genotypes and *qrt* mutant genotypes, and were screened by PCR. Selected F2 generation plants, *apy1apy1; APY2APY2; qrtqrt* and *APY1APY1; apy2apy2; qrtqrt*, were crossed to produce the F3 generation. All the plants in this generation were heterozygous for both apyrases and homozygous for *qrt* (*APY1apy1; APY2apy2; qrtqrt*) and, consequently, all the pollen showed the tetrad phenotype. F4 generation plants are the progenies that are produced by pollinating single knockout lines carrying *qrt* allele with pollen produced by double knockout heterozygous plants with *qrt* mutant. Plants with genotype of *APY1apy1; apy2apy2; qrtqrt* were screened by PCR in the F5 generation.

Scanning electron microscopy

Flowers at stages 13 or 14 were picked from the plants and dried on the bench at room temperature for 24 hours. Dried pollen grains were dipped onto stubs covered by double-sided mounting tapes. Pollen grains were sputter coated with gold and viewed with a scanning electron microscope (Philips EM 515) at an accelerating voltage of 5 kV.

Construction of APY1-GFP fusion protein and onion bombardment

Full length *APY1* coding region lacking the stop code were amplified by PCR with primers APY1F-EcoRI and APY1R-GFP to produce *APY1-G* with a restriction enzyme site of EcoRI at the 5'-end. A BamHI site was added at the 3'-end of *GFP* genes in PCR by GFP-R primer. The *GFP* gene was amplified by GFP-F and GFP-R to produce *A-GFP*. 30 bp overlaps were produced at the 3'-end of *APY1-G* and 5'-end of *A-GFP* including a 15 bp linker sequence between the *APY1* and *GFP* genes. The PCR product *APY1-GFP* was produced by using *APY1-G* and *A-GFP* as the template and amplified by primers APY1F-EcoRI and GFP-R. *APY1-GFP* was subcloned into the pCR2.1-TOPO vector to produce pAPY1-GFP. Sequencing of the pAPY1-GFP showed that there were no errors in the *APY1-GFP* gene. The *APY1-GFP* fragment was released from pAPY1-GFP by EcoRI and BamHI, and inserted into the pHANNIBAL vector digested with the same restriction enzymes to produce pHA-APY1G.

Particle bombardment was used to transiently express *APY1-GFP* in onion epidermal cells. Onion (*Allium cepa*) epidermal layers were pulled off from onion leaves by a pointed forceps. The strips of the epidermis were placed on MS agar plates. The pHA-APY1G construct was isolated using the Plasmid Maxi Kit (Qiagen). Gold

particles of 1.6-µm were coated with plasmids and bombarded into the onion epidermal layers at 1100 psi by a Bio-Rad Pds1000/He gun as described by Weigel and Glazebrook (2002). After bombardment, the petri dishes were sealed with parafilm and the onion cells were incubated at room temperature in darkness for 16 hours. Onion cells were observed by a Leica DM IRBE microscope. Plasmolysis of onion cells was performed by placing the onion cells into 0.5 M Mannitol for 3 min. Images were acquired by a Lieca DFC350 FX fluorescence camera.

Primers used

APY1F-EcoRI: 5'- GAATTCATGACGGCGAAGCGAGCGATCGGAC -3'

APY1R-GFP: 5'-CACCATTCCTGCTGCGCCTCCTGGTGAGGATACTGCTTCT -3'

GFP-R: 5'-GGATCCTTACTTGTACAGCTCGTCCATGCCG-3'

GFP-F: 5'-TCCTCACCAGGAGGCGCAGCAGGAATGGTGAGCAAGGGCG-3'

RESULTS

Double knockout *apy1apy2* pollen fails to germinate

To investigate the possibility that the double knockout of *APY1* and *APY2* causes male sterility, pollen grains were colleted from flowers of plants heterozygous for both apyrases, and germination was tested *in vitro*. Because the absolute germination rates varied greatly from one experiment to another (Fig. 2.1, A-C), data from independent experiments were not pooled. Environmental and handling conditions are difficult to

reproduce exactly for each experiment, but are more similar for pollen of the same experiment. Therefore, only germination rates from pollen germinated in parallel in the same 16 hr time period were compared.

According to Mendelian laws, approximately 25% of the pollen produced by these plants would be expected to be double knockout. The average germination ratio of heterozygous pollen was 63.4% ± 1.7% (Fig. 2.1 A), which was about 75% of wild-type controls. This 25% reduction coincided with the expected percentage of double knockout pollen. In single heterozygous plants, *apy1apy1*; *apy2APY2* and *apy1APY1*; *apy2apy2*, the germination rate was reduced to 28.7% ± 2.0% and 34.8% ± 0.5%, respectively (Fig. 2.1 B). This represented a 50% lower germination rate compared with the wild-type value of 60.5% ± 4.5%. This reduced germination rate again matched the expected value if double KO pollen did not germinate. As an additional control, the germination rate of pollen from single knockout lines was determined in comparison with pollen from wild-type, and no significant difference was found (Fig. 2.1 C). Taken together, the results suggest that the lack of both apyrases in Arabidopsis pollen may prevent germination.

The vast majority of pollen carrying the mutation display normal pollen development

Pollen grains from double heterozygous plants were analyzed for morphological and nuclear aberrations as well as viability. Roughly one-fourth of this pollen was expected to carry the *apy1apy2* genotype. DAPI (4',6-diamidino-2-phenylindole) staining for double-stranded DNA (Coleman et al. 1985), showed that 94% ± 3.1% of the tested

pollen was identical to the wild-type pollen, which has two sperm nuclei and one large vegetative nucleus in each pollen grain (Fig. 2.2 E, F). In pollen, aberrant divisions may lead to changes in cell fate and polarity (Park et al. 1999), however, the size, shape and polarity of most pollen grains from plants heterozygous for both apyrases were not different from the wild-type controls (Fig. 2.2 G, H).

It is possible that double knockout pollen fails to germinate because pollen lacking apyrase are not viable. Fluorescein diacetate, which can freely diffuse across the cell membrane where it is cleaved by endogenous esterase activity of living cells producing a green fluorescent product, may be used as a viability assay. Using this assay, about 98% of the heterozygous pollen looked viable and had an integral plasma membrane (Fig. 2.2 A, B). Therefore, apyrases may not affect pollen viability but might play an important role during pollen germination.

Apyrase inhibitors inhibit pollen germination in a dose-dependent manner

Apyrase inhibitors are chemical compounds that can inhibit apyrase activities at low concentrations. Several apyrase inhibitors were screened and characterized by Windsor et al. (2002). However, the efficiency of apyrase inhibition is different among the inhibitors, and all of the inhibitors can also non-specifically inhibit the activities of alkaline and acid phosphatases with varying efficiencies. In our experiments, apyrase inhibitor NGXT13 was selected for its high efficiency in blocking apyrase activity (100%) but minimal non-specific inhibition of alkaline phosphatases (8%) and acid phosphatases (27%). The data showed that addition of apyrase inhibitor NGXT13 to liquid germination medium can completely block or significantly inhibit pollen

germination at concentrations of 5.25 µM and 2.1 µM, respectively (Fig. 2.3). 1.55 µM, pollen can germinate with no significant difference when compared to wild-type germination rates. This result showed that by adding an apyrase inhibitor, pollen germination was also inhibited, which is similar to the result for mutant pollen that carries the double knockout for both apyrases.

Mutant pollen shows reduced pollen germination rate and malformed grains in tetrad analysis

Since the conclusions we drew on apyrase involvement in pollen germination were indirect, based on statistical evaluation of germination rates in pollen populations, more direct evidence that the pollen that do not germinate are truly double knockout gametes was needed. In Arabidopsis quartet (*qrt*) mutants, the four meiotic products of the pollen mother cell are fused together because the cell loses the ability to degrade the pectin component, making the pollen tetrad intact. Tetrad analysis, which makes it possible to study the four products of a single meiotic event in *qrt* mutants, was chosen to assay Arabidopsis pollen development in double knockout pollen (Preuss et al. 1994). Tetrad pollen collected from *APY1apy1; apy2apy2; qrtqrt* were germinated *in vitro*. Since two out of four pollen in one tetrad are double knockout pollen, based on previous results, we predicted that heterozygous mutant pollen would only have two germinating pollen. The results were scored by number of pollen germinated in each individual tetrad group. In control pollen (CS8846), the germination was distributed in a broad range from 0 to 4. However the mutant only fell into 0, 1, and 2 pollen grains

germinating (Fig. 2.4 A). This result provided further evidence that double knockout pollen is defective in germination.

Malformed pollen grains were detected in pollen produced by *APY1apy1; apy2apy2; qrtqrt* mutants by scanning electron microscopy (SEM) (Fig 2.4 B). In a single tetrad pollen two out of four pollen were misshaped pollen grains, which are presumably the two double knockout pollen, and another two pollen showed indistinguishable morphology from the control. Over 95% of the quartet pollen produced by *APY1apy1; apy2apy2; qrtqrt* mutants showed malformed pollen grains. This was only apparent in the pollen examined by SEM, which are extensively air dried prior to analysis, but not those examined by regular light microscopy, which are not subjected to extensive drying. This is possibly due to the hypersensitivity of the double knockout pollen to the highly dehydrated environment after extended dehydration time.

Effects of apyrase antibody and apyrase inhibitors on wild-type pollen tube growth

This work was done in collaboration with an undergraduate student in our lab, Jonathan Torres. Polyclonal anti-apyrase antibodies that block apyrase activity *in vitro* were tested for their effects on wild-type pollen tube elongation. Tests of the specificity of these antibodies by Western analyses showed that they could specifically bind to APY1 and APY2 but not other proteins. Pollen tube growth was dramatically decreased after application of the immune serum of anti-apyrase antibody. The pollen tubes treated by preimmune serum showed no statistically different growth rate from the control treated only with pollen germination media (Fig. 2.5 A). Thus the decreased growth of pollen tubes treated with apyrase antibody was likely due to the binding of the

antibody to its antigen and the inhibition of apyrase activity. The amount of immune serum used in this study was 0.4 μg and 0.8 μg total protein and both concentrations significantly inhibit pollen tube growth. The level of inhibition was increased with the increasing concentration of immune serum. Consistent with these results, two previously characterized apyrase inhibitors (Windsor et al., 2002) (Texagen Inc. Austin, TX) were selected to assay the effects of apyrase inhibitors on pollen tube growth. Both inhibitors inhibited pollen tube growth at a significant level ($p \leq 0.01$) (Fig. 2.5 B).

Transient gene expression of APY1-GFP in onion epidermal cells

To investigate the subcellular localization of apyrases, the *APY1* gene fused with a GFP tag was generated. This fusion protein was driven by a constitutively expressed promoter--cauliflower mosaic virus (CaMV) 35S promoter. The *35S-APY1-GFP* gene and the control gene *35S-GFP* were transiently expressed in onion epidermal cells. Different from the control, in which the GFP signal was always distributed in cytoplasm, the *35S-APY1-GFP* protein was located mainly on the cell periphery with some in cytoplasm (Fig. 2.6). After treatment with 0.8 M mannitol, cells underwent plasmolysis. The GFP signal was pulled away from the cell wall with the plasma membrane, which indicated that the tagged apyrases were not located in cell walls. However, tests of whether the APY1-GFP can complement the apyrases knockout phenotype have not yet been done. Thus, the localization of apyrases needs further investigation.

DISCUSSION

Two Arabidospsis apyrases were previously cloned and genetic studies of T-DNA insertional mutants were used as an approach to elucidate the physiological functions of these two apyrases (Steinbrunner et al. 2000, 2003). During the attempt to overcome potential gene redundancy by generating double knockout (DKO) lines, the absence of the *apy1apy2* genotype was discovered. A functionally defective gametophyte was identified, because the germination rate of pollen from double heterozygous mutant plants and from mutant plants containing only one wild-type apyrase gene was reduced by a percentage that correlated with the fraction of pollen genetically expected to bear the double knockout genotype.

There are three possible reasons for the defective pollen germination in *apy1apy2* pollen: 1) Pollen carrying the mutant allele became non-functional in a certain phase of pollen development after meiosis; 2) The lack of apyrases affected pollen viability; 3) Mutant pollen could germinate much slower than other pollen carrying at least one of the apyrases during the process of pollen germination. To address these possibilities, the numbers and size of nuclei, pollen polarity, and pollen viability were tested.

In wild-type pollen, a characteristic feature of normal developed pollen was the presence of three nuclei--one from the vegetative cell and two from the sperm cells. Defective development of these nuclei can result in a different cell fate determination with the consequence of abolished pollen germination (Park et al. 1998, Chen and McCormick, 1996). Our analyses of the numbers of nuclei in mature pollen grains by DAPI staining using apyrase heterozygous pollen revealed that the numbers and

morphology of DKO pollen nuclei were the same as observed in wild-type pollen. The analyses of pollen polarity also showed no difference between DKO pollen and wild-type pollen. Therefore, the pollen development in mutant pollen was normal.

The inhibition of germination of DKO pollen could not be simply accounted for by death. The viability assay performed tested for plasma membrane integrity and metabolic activity (Heslop-Harrison and Heslop-Harrison, 1970), both signs of vital cells. This eliminated two possible reasons that might cause aborted germination. Thus apyrases could be involved in the stage that initiates pollen germination.

The lack of germination of apyrase-deprived pollen, alternatively, could be explained by a very slow germination process. Pollen tubes of the *tip1-2* mutant, for example, grow much more slowly than wild-type (Ryan et al. 1998). If the pollen tubes of *apy1apy2* grains emerged at a much later time point than wild-type, the pollen would appear not to have germinated. However, in wild-type pollen, germination is usually visible within 15 min of initiation (Pruitt and Hülskamp 1994), and germination studies are generally evaluated after a 4- to 6-h time period. In this study, the window of germination was extended to a minimum of 16 hours, which makes lack of time a very unlikely reason for the absence of germination.

The determination of transmission efficiency of the *apy1apy2* allele was examined by reciprocally backcrossing with wild-type and showed the mutant allele is not transmitted through the male gametes but could be transmitted through the female gametes (Steinebrunner et al. 2003).

The inhibition of pollen germination by inhibiting apyrase activity demonstrated that the activity of the enzyme, not just the activity of the gene, was necessary for pollen

germination. Although the apyrase inhibitor used was originally characterized only *in vitro* (Windsor et al. 2002) and could exhibit toxic effects on a cell unrelated to its effects on apyrase. Viability assays indicated that the pollen remained metabolically active, although ungerminated, after it was treated with the inhibitor (data not shown). Thus the inhibition of germination of wild-type pollen could not be simply accounted for by death.

In Arabidopsis *qrt1* mutants, the four meiotic products of the pollen mother cell are fused together due to the disrupted gene function of QRT1, which is a pectin methylesterase gene. The *qrt1* pollen loses the ability to degrade the pectin component, and this leaves the pollen tetrad intact (Preuss et al. 1994). Tetrad analysis, which can be used to study the four products of a single meiotic event in *qrt* mutants, was used to assay pollen development in *apy1apy2* mutant. Throughout the tetrad analysis the affected pollen germination was observed as expected. This provided further evidence to suggest that knocking out apyrases resulted in a gametophytic mutation. Misshapen DKO pollen grains were only found in tetrad analysis using SEM. The collapse of the DKO pollen was most likely caused by the prolonged dehydration time, which is essential for samples used in SEM, and may have been due to some defect in the cell wall of the mutant pollen.

Two yeast apyrases, GDA1 and YND1, localized to the Golgi membrane are involved in controlling the *N*- and *O*-linked glycosylation in yeast. The double mutant of these two genes showed defects in cell integrity and cell growth possibly due to the essential role of hydrolysis of GDP to GMP in mannan synthesis, which is one of the

basic compounds of the cell wall (Gao et al. 1999). The Arabidopsis apyrases in pollen may function similarly as yeast apyrases and this requires further study.

The expression of APY1 and APY2 is abundant not only in pollen but also in pollen tubes and other organs (Steinebrunner et al. 2003, Wu et al. 2007). Chemical inhibitors that can mimic the effects of apyrases on pollen germination and polyclonal antibodies that recognize both apyrases were used to specifically inhibit the enzyme activity of apyrases during pollen tube elongation. As expected, pollen tube growth was inhibited by the addition of anti-apyrase antibody or apyrase inhibitor into the growth medium. The inhibition of pollen tube growth was correlated with the raised concentration of ATP in growth medium (Wu et al. 2007). This showed that the activity of apyrases plays a significant role in controlling the equilibrium concentration of ATP in the extracellular space in pollen tube elongation.

Both APY1 and APY2 proteins have predicted signal peptides by protein structure analysis (Steinebrunner et al. 2000). The observation of pollen tube growth inhibition by anti-apyrase antibody and chemical inhibitors indicated that Arabidopsis apyrases might be functioning as ectoapyrases. This is further confirmed by transient expression of APY1 in onion epidermal cells and the immunoblot analysis of plasma membrane-enriched fractions with apyrase antibody (T. Butterfield, Master Thesis).

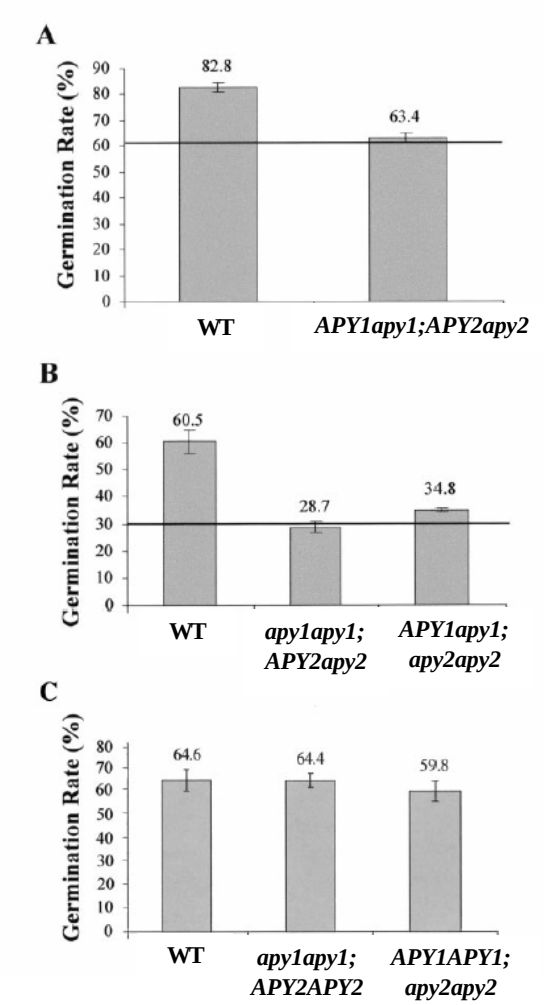

Figure 2.1 Reduction in pollen germination rate by the percentage of expected double KO genotypes, and maintenance of wild-type germination rates in single KO pollen.

The germination rates were calculated as the number of germinating pollen divided by the number of pollen sown multiplied by 100. The horizontal line marks 75% (A) and 50% (B) of the average wild-type germination rate, respectively. Bar graphs represent means with SDs from triplicate assays (wild-type pollen) and from quadruplicate assays (pollen from double heterozygous plants; A) and from triplicate assays (B and C). For each assay the number of pollen grains counted was more than 300. (This figure was published in Steinebrunner et al. 2003.)

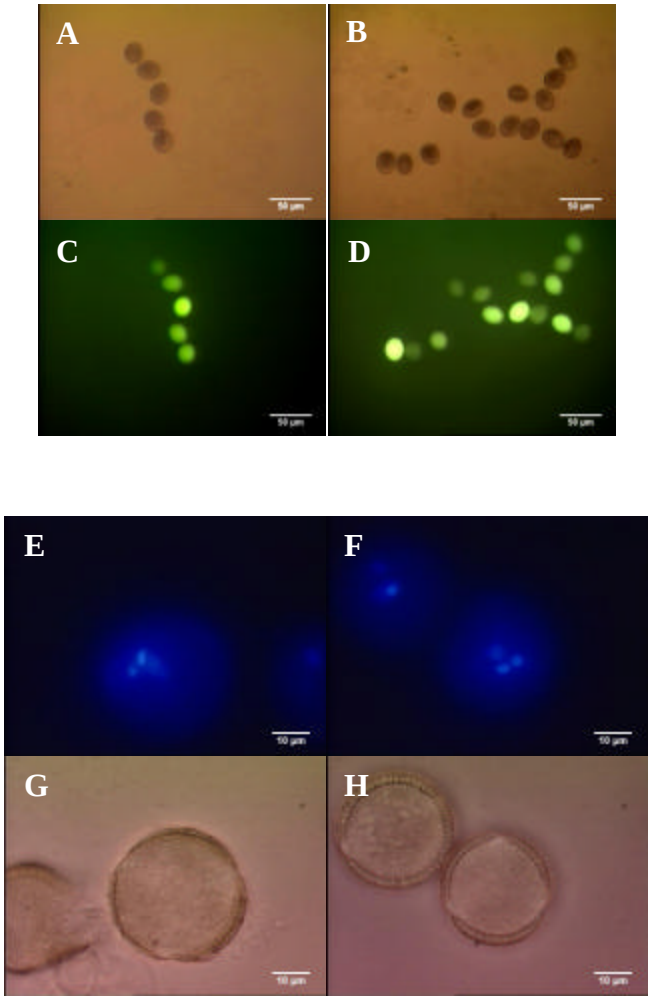

Figure 2.2 Viability and cytochemical analyses of double KO pollen.

Pollen viability assay using fluorescein diacetate showed that double KO pollen produced from *apy1apy1;APY2apy2* (A, C) is as viable as wild-type pollen (B, D). Bars = 50 µm (A to D). Nuclei formation and pollen polarity analyses were also examined by DAPI staining and light microscopy. Double KO pollen produced from *apy1apy1;APY2apy2* (E, G) displayed indistinguishable phenotype from wild-type control (F, H). Bars = 10 µm (E to H). At least 50 pollen grains were examined in each experiment. (This figure was published in Steinebrunner et al. 2003.)

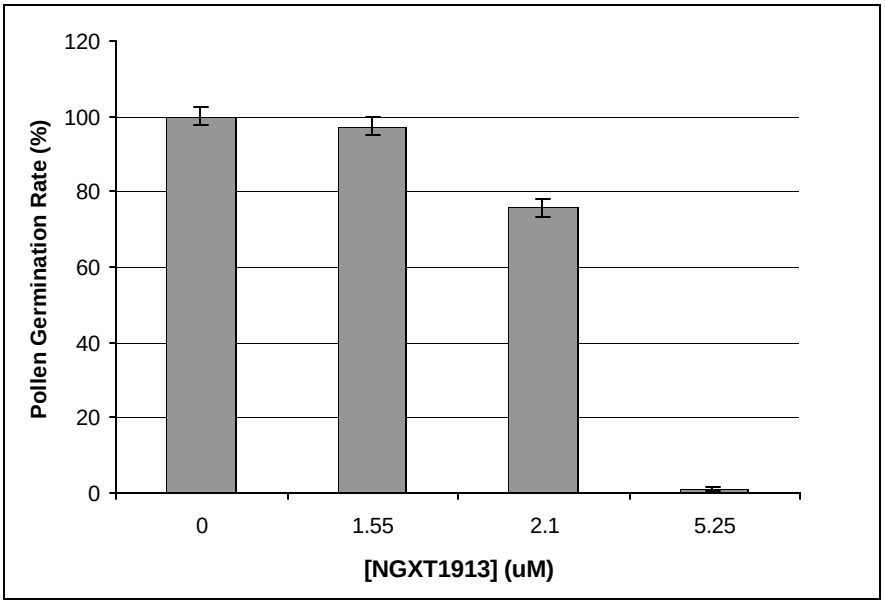

Figure 2.3 Inhibition of apyrase activity by apyrase inhibitor NGXT 1913 inhibit pollen germination.

To test whether apyrase activity was necessary for pollen germination, wild-type pollen was treated with NGXT 1913, a compound shown to be a strong inhibitor of apyrase activity. The pooled results of four separate experiments (each performed in duplicate) showed that an inhibitor concentration of 5.25 μM almost completely blocked germination; 2.1 μM significantly reduced germination to 75.8% ± 2.3% of control levels ($P < 0.05$, Student's *t* test); and 1.55 μM did not affect the germination rate in a statistically significant way compared with untreated pollen. Error bars are ± SD. (This figure was published in Steinebrunner et al. 2003.)

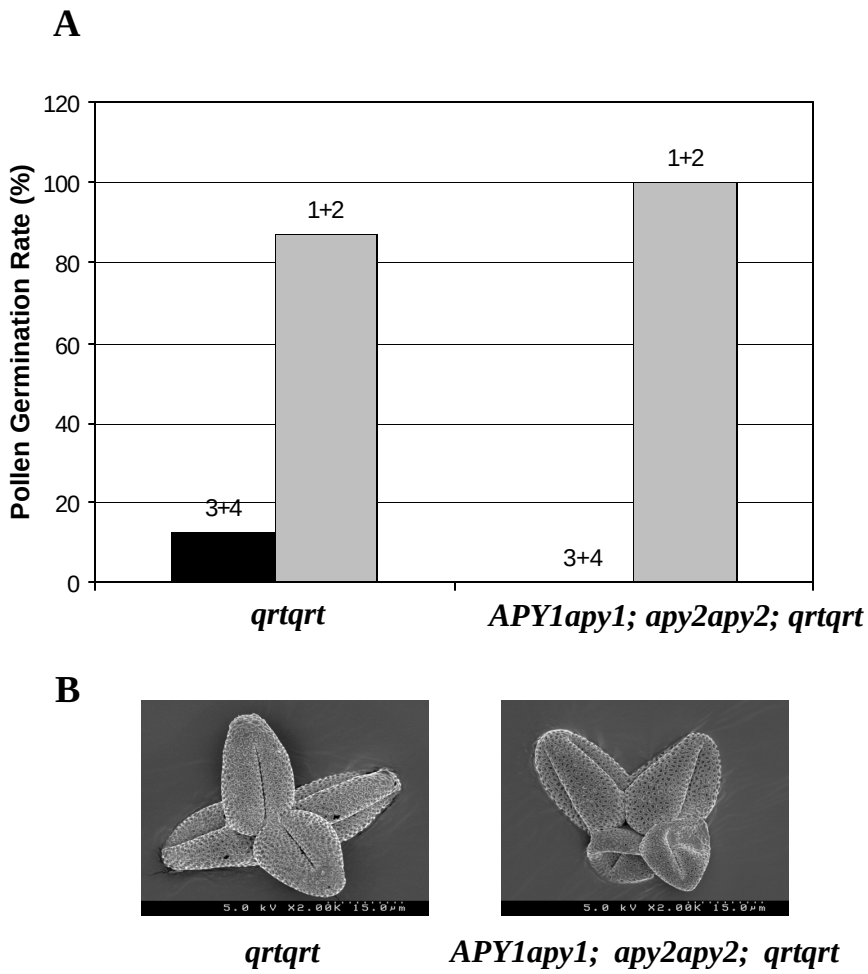

Figure 2.4 Tetrad analysis of double KO pollen.

Tetrad analysis of double KO pollen was performed using pollen from *APY1apy1;apy2apy2;qrtqrt* and *qrtqrt* mutant. The pollen germination rate of *qrtqrt* mutant and *APY1apy1;apy2apy2;qrtqrt* were scored by 3 or 4 germinated pollen and 1 or 2 germinated pollen from one tetrad pollen (A). No tetrad with 3 or 4 pollen germinated was found in *APY1apy1;apy2apy2;qrtqrt* mutant pollen. Scanning electron microscopy image showed that 2 out of 4 pollen in one tetrad exhibited a malformed phenotype in *APY1apy1;apy2apy2;qrtqrt* pollen.

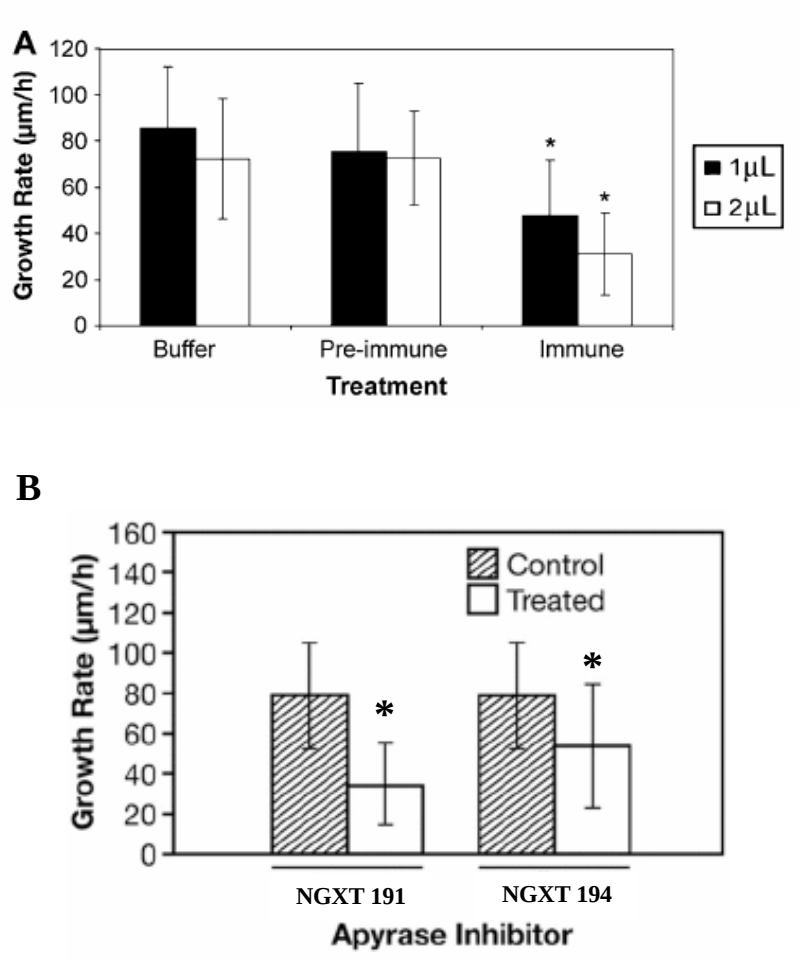

Figure 2.5 Inhibition of apyrase activity in pollen tubes by antibodies and apyrase inhibitors decrease tube elongation.

Pollen tubes treated with polyclonal anti-apyrase antibodies displayed rapid decreases in growth rate. Error bars are ±SD and asterisks mark growth rates that are significantly different from that of the buffer control ($P \leq 0.02$; $n \geq 20$, Student's t test). The differences in growth rates of tubes treated with pre-immune serum and of tubes treated with buffer are not statistically different ($P > 0.6$; $n \geq 20$). Protein concentration of the pre-immune sera was 0.3 μg/μL, and of the immune sera was 0.4 μg/μL (A). The application of apyrase inhibitors NGXT 191 and NGXT 194 in pollen germination medium during tube elongation showed that both inhibitors inhibited tube growth at significant level. Error bars are ±SD and asterisks mark growth rates that are significantly different from that of the control ($P \leq 0.01$; $n > 20$, Student's t test) (B). (This figure was published in Wuet al. 2007.)

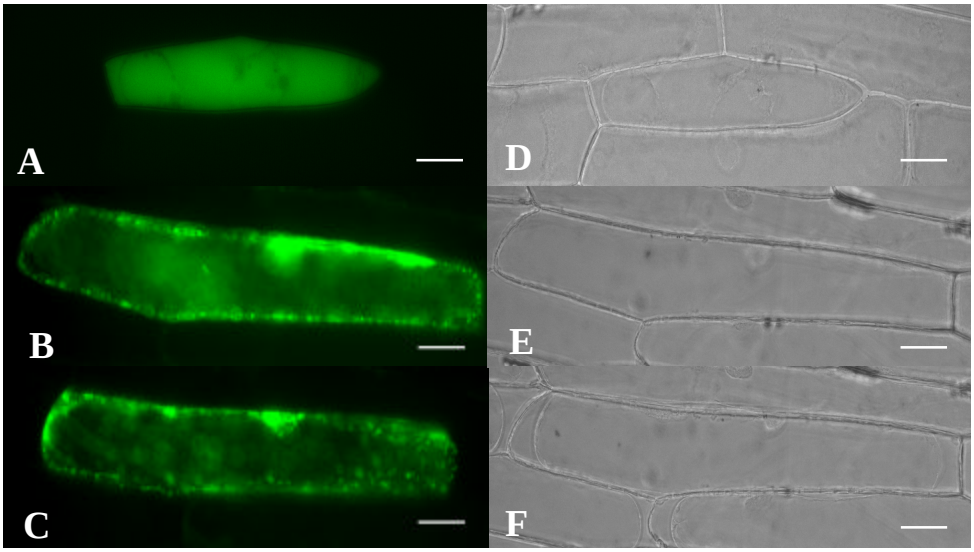

Figure 2.6 Transient expression of APY1-GFP fusion protein in onion epidermal cells.

As a control, the expression of GFP only in onion cells was observed and found to be diffuse (A, D). The *GFP* gene was driven by a *35S* promoter in the same vector as the *APY1-GFP* (A, D). Cells expressing APY1-GFP fusion protein showed localization on cell periphery and in the cytoplasm (B, E). Plasmolysis induced by 0.8M Mannitol indicated that the localization of APY1-GFP was not in cell wall (C, F). D to F were bright-field images corresponding to images on top. Bars = 50μm.

CHAPTER 3: APYRASE EXPRESSION IS STRONGLY CORRELATED WITH GROWTH IN ARABIDOPSIS

INTRODUCTION

Previous results using promoter-GUS analysis showed that *APY1* and *APY2* are strongly expressed in areas undergoing rapid growth and active secretion, such as root tips in both primary and lateral roots, etiolated hypocotyls, and pollen tubes. In pollen tube elongation the suppression of apyrases is correlated with growth suppression (Wu et al. 2007). These data suggest that the function of apyrase may correlate with growth control.

Plant growth is regulated by many factors. Light, as one of these factors, can induce the germination of seeds and also modulate the growth after seeds germinate. When the light condition changes, this can cause rapid growth changes in different areas of the whole seedling. When plants are exposed to light, the primary root, which emerges first from the seed coat during seed germination, is stimulated to grow faster, and it orients downward away from light thus giving it greater access to the water and nutrients in soil. In contrast to light's stimulation of root growth, light suppresses the growth of hypocotyls. As seen in etiolated seedlings, the effects of darkness are opposite to those of light: hypocotyl elongation is stimulated, and root growth is inhibited.

The expression of APY1 and APY2 in Arabidopsis is regulated by light at both the transcript and protein level. The red light signals that are known to depress the

growth of hypocotyls can even more rapidly induce the disappearance of apyrase protein in this tissue within 3 min after a 4 min irradiation (Wu et al. 2007).

Auxin is a growth hormone that plays a crucial role in regulating plant growth and development. Indole-3-acetic acid (IAA), which is recognized as the most common form of auxin in plants, can be detected in varieties of organs, such as leaves, cotyledons, and roots (Ljung et al. 2001), but young apical leaves are the main source for producing auxin for the whole seedling. The movement of auxin to other tissues is mainly through a directional transport system called polarized transport by membrane and carrier-mediated transport between cells (Delbarre et al. 1996, Noh et al. 2001). The transport of auxin controls many aspects of root growth and developmental processes including primary root development, gravity responses, and initiation of lateral roots (Muday 2001, Marchant et al. 2002).

In Arabidopsis root tissues, shoot-derived IAA was delivered to the root apex through polar transport, which moves auxin through central stele cells in the roots, and through phloem-mediated transport. Ljung et al. (2005) reported that for four-d-old seedlings both transports are equally important, whereas phloem-mediated transport of auxin becomes dominant in eight-d-old seedlings. The high concentration of auxin that accumulates in root tips on reaching the root apex is transported backwards (basally) to the lateral cap and epidermis (Jones 1998). Thus root transport of auxin involves two distinct polarities—acropetal transport and basipetal transport.

Genetic studies of auxin polar transport reveal there are several protein facilitators that regulate the auxin influx and efflux in cells. Arabidopsis *PIN*-type genes control the efflux of auxin, and mutants of *PIN* genes show disrupted development processes

(Paponov et al. 2001). PIN proteins are asymmetrically distributed along the cells and result in an uneven transport of auxin in tissues (Friml 2003). In roots, PIN1 proteins were found abundantly on the basal membranes of the stele cells to facilitate the movement of auxin toward the root apex (Gälweiler et al. 1998). PIN2 and PIN3 proteins are localized in the membrane of columella cells and other gravity-sensing cells such as starch sheath (Friml et al. 2002, Müller et al. 1998). In Arabidopsis the *PIN2* mutant has altered auxin transport, and this change is correlated with reduced root elongation and deficient gravitropism responses (Müller et al. 1998). Besides PIN proteins, two members of multidrug-resistance type ATP-binding cassette (ABC) family, MDR1 and PGP1, were identified as being critically needed for normal auxin efflux (Noh et al. 2000). There are also transport proteins that are specialized for moving auxin into cells; i.e., there is an auxin influx apparatus. AUX1 was identified as an auxin influx facilitator localized primarily on the side of cells opposite to that of PIN1, and it, like PIN1, is essential for transporting auxin from leaves to roots (Swarup et al. 2001). The *aux1* mutant showed a reduction of lateral root number and less accumulation of auxin (Marchant et al. 2002). This revealed that AUX1 was important for transporting auxin from source to sink tissues and later for redistributing it from the root apex to more basal regions of the root. The mobilization of auxin is mediated by different auxin transporters and this carrier-mediated transport is essential to trigger the new organ development and plant growth.

Tang et al. (2003) reported that disruption of auxin transport was observed in root tips of Arabidopsis seedlings treated with exogenous ATP in the growth medium. The effect of auxin accumulation in the root tip caused by 2 mM ATP is similar to treatments

with NPA, an auxin transport inhibitor. Addition of ATP to the growth medium also resulted in the hypersensitivity of wild type seedlings to growth inhibition by exogenous auxin (Tang et al. 2003). But what mechanism links ATP and auxin together is still unclear and needs further study. In this chapter we discuss the important role of apyrases in growth control in plants, and provide data indicating that one way they could do this is by maintaining concentrations of extracellular ATP released during growth below growth-inhibitory levels. Correspondingly, we propose that a possible reason why apyrase DKOs have suppressed growth is because in these mutants [eATP] rises to a level sufficient to inhibit normal auxin transport.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis thaliana ecotypes Columbia (CS907) and Wassilewskija (WS) were used as wild types in this study. Seeds were surface sterilized and planted on Murashige and Skoog (1962) medium (4.3 g/L Murashige and Skoog salts (Sigma), 0.5% MES, 1% sucrose, and 1% agar, pH 5.7 with 5M KOH). The *apy1* and *apy2* mutants were isolated previously (Steinebrunner et. al., 2003). For root and hypocotyl growth assays, seeds were sown on the surface of germination medium. Plates were placed upright in a culture chamber and grown at 23° C under 24 hours fluorescent light. For the root growth assay images were taken every 24 hours on day three through day six with a Nikon Coolpix990 digital camera. For measurements of the growth of etiolated

hypocotyls, plates with sown seeds were wrapped in aluminum foil and placed in a growth chamber for 3.5 d, then unwrapped under white light and photographed immediately.

Generation of overexpressing lines and plant transformation

To generate *35S:APY1-Myc* lines the cDNA region of *APY1* was amplified by the primers Apy-a and Apy-b to produce PCR fragment *APY1-M* (containing the first 21 bp of *Myc* at 3' end). Six copies of the *Myc* epitope tag were amplified by primers Myc-c and Myc-e to produce a PCR product *A-Myc* (containing the last 20 bp of *APY1* at 5' end). *APY1-Myc* was generated by mixing *APY1-M* and *A-Myc* together and amplifying by primers Apy-a and Myc-e. The PCR product was subcloned into the pCR2.1-TOPO vector (Invitrogen) to generate pTOPO-APY1. The *APY1-Myc* fragment was sequenced and cut with EcoRI. The released insert was then ligated into EcoRI site of the pLBJ21 binary vector, which contains the 35S promoter of cauliflower mosaic virus. This construct was introduced into the *Agrobacterium tumefaciens* strain GV3101 that was used to transform Columbia wild-type by the vacuum infiltration method (Clough and Bent, 1998). Twenty transgenic lines containing the construct were selected with 50 µg/ml kanamycin on germination plates. Plants with resistance were selected and transplanted to soil. T2 seeds from individual T1 plants were screened to generate homozygous and single locus insertion lines.

Generation of the RNAi construct and plant transformation

To generate the RNAi construct the sense cDNA region containing the 220-bp near the 3' end of *APY1* was amplified by primers APY1-XhoI and APY1-SalI. The antisense region was amplified by primers RNAiI-EcoRI and RNAiI-SpeI. The PCR products of sense and antisense fragments were sequenced and subcloned into pSKint in the sense direction by XhoI and SalI and in the antisense direction by EcoRI and SpeI. The fragment containing the sense, an actin 11 intron, and the antisense sequences was cut by XhoI and SpeI. The released fragment was used to replace the original GFP-RNAi fragment in the pX7-GFP binary vector to produce pX7-APY1. The pX7-GFP binary vector and pSKint were provided by Dr. Nam-Hai Chua, Rockefeller University (Guo et al. 2003). The pX7-APY1 binary vector was electroporated into *Agrobacterium tumefaciens* strain GV3101. The *apy2* mutants were transformed by pX7-APY1 via a floral dip method (Clough and Bent, 1998). Transgenic plants were selected on MS plates containing 20 μg/ml hygromycin (Sigma). Homozygous and single locus insertion lines were selected by examining the resistance for hygromycin in T2 seeds.

To induce expression of the RNAi constructs, the transformed plants were either germinated and grown on agar in media containing 4 μM estradiol (Sigma), or germinated and grown on soil that was watered at regular intervals with 4 μM estradiol in dd H_2O water. The aerial parts of plants grown on soil were also sprayed with 4 μM estradiol whenever they were watered.

RNA gel blot analysis

Seven-d-old Arabidopsis seedlings were collected and frozen in liquid nitrogen. Total RNA was isolated using the RNeasy Plant Mini Kit (Qiagen). Ten micrograms of

RNA were separated in a 1.2% (w/v) agrose gel with 6% (v/v) formaldehyde. The RNA was transferred to a Zeta-Probe GT Membrane (BIO-RAD) and hybridization was performed according to the manufacturer's instructions.

Assay of lateral root formation and numbers of adventitious roots

Fourteen-d-old light grown seedlings were used to detect lateral root formation in RNAi lines. As control plants *apy2* single knockout mutants were used. Both control and RNAi lines were planted in the medium containing 4 µM estradiol to induce the RNAi silencing. The control and the RNAi lines were grown in one 150 mm petri dish to assure identical growth conditions. The experiment was repeated three times. The n value for each RNAi line and control was around 30. The numbers of adventitious roots were counted on the same day with a dissection microscope.

Scanning electron microscopy

Fresh six-d-old light grown seedlings were fixed in 0.1 M phosphate buffer solution (pH 5.7) containing 1% paraformaldehyde and 2% glutaraldehyde. Samples were fixed under vacuum for 30 min and incubated at 4ºC for 12-24 hours. After about 20 h fixed seedlings were washed three times in phosphate buffered saline (pH 7.4), followed by two times in distilled water for 10 min. Samples were dehydrated at room temperature in an ethanol series for 15 min at each step as follows: 15%, 30%, 50%, 70%, 80%, 90%, 95%, 2x absolute ethanol. Dehydrated specimens were further dried by critical point drying and mounted onto the stubs using double-sided mounting tapes. Dry seedlings were sputter coated with gold immediately after critical point drying.

Images were captured by a Philips EM 515 scanning electron microscope operating at an accelerating voltage of 14.7 kV.

Generation of DR5-GFP RNAi lines

To generate the DR5-GFP RNAi lines, the R2-4A line was crossed with a transgenic line expressing DR5-GFP. The F1 generation plants were selected on MS medium containing 20 μg/ml hygromycin. The plants that grew on hygromycin plates are heterozygotes of *APY2*, RNAi gene, and DR5-GFP gene, but homozygotes of *APY1*. The seeds harvested from the F1 generation plants were planted on agar in media containing 4 μM estradiol in 150 mm petri dishes. After 6 days of growth in light, seedlings that showed decreased root growth were selected and fluorescence images were captured by a Leica SP2 AOBS confocal microscope.

Primers used

Apy-a: 5'-ATAGAATTCATGACGGGGAAGGGA-3'

Apy-b: 5'-ATCGATACCGTCGACCTCGAGTGGTGAGGATACTGCTTCT-3'

APY1-SalI: 5'-ATAGTCGACGTATTTCACCTTCTT-3'

APY1-XhoI: 5'-ATACTCGAGAAACCAACCTGTGGC-3'

Myc-c: 5'-AGAAGCAGTATCCTCACCATCTCGAGGTCGACGGTATCGA-3'

Myc-e: 5'-GTATCATTCATTCAGTCAAAAGTCCTC-3'

RNAiI-EcoRI: 5'-ATAGAATTCGTATTTCACCTTCTT-3'

RNAiI-SpeI: 5'-ATAACTAGTAAACCAACCTGTGGC-3'

RESULTS

Overexpression of apyrase enhances growth

To further study the function of apyrases in Arabidopsis, overexpression lines of apyrases were generated. Previous results using promoter-GUS constructs, which were driven by the promoter regions of either *APY1* or *APY2*, suggested high expression level of apyrases in etiolated hypocotyls between days one and five for both GUS fusions, and this expression was suppressed by light. In etiolated seedlings the overexpressing lines of *APY1* and *APY2* were analyzed by measuring the hypocotyl length at 3.5-d. Overexpressing *APY1* resulted in a 15% increase in growth over wild type control (Fig 3.1 A), but overexpressing *APY2* did not show increased growth (data not shown). Under continuous light conditions, the growth of wild type plants and plants overexpressing *APY1* or *APY2* was not different.

Single knockouts of either apyrase result in decreased growth

apy1 and *apy2* single-knockout mutants were screened previously from a T-DNA insertion pool. *apy1* mutant line A1J1 and *apy2* mutant line A2-11 were identified as null mutants by semi-quantitative RT-PCR, and further analyses of mutant phenotypes were performed under different conditions. There were no obvious phenotypes detected at that time (Steinebrunner et al. 2003). However, the measurement of the etiolated hypocotyls of these single knockout mutants was not performed at this time.

Based on the results obtained from overexpression lines, which showed the increased hypocotyl length, the hypocotyl lengths of *apy1* and *apy2* single mutants were examined. In both mutant lines more than 100 etiolated 3.5-d-old seedlings were

measured. The hypocotyl elongation assay of *apy1* and *apy2* mutant plants showed about 15% reduced lengths compare to wild type after 3.5 days growth in the dark ($P < .01$) (Fig. 3.1 B).

Suppression of the expression of APY1 and APY2 also suppresses root and shoot growth

To further assess the effects of suppression of *APY1* and *APY2* on growth, we carried out this suppression by inducing an apyrase-directed RNAi construct in *apy2* plants that were wild type for *APY1* but homozygous for the *apy2* knockout mutation. The RNAi construct was made by inserting a sense and an antisense region of *APY1* cDNA (132 bp) into the vector with an intron in between. The structure of this construct predicts that when estradiol is applied to transformed plants harboring the RNAi construct, the hormone will induce the production of the sense-intron-antisense mRNA, which will form a hairpin structure, making it a target for breakdown by the RNAi machinery of the cell. The small pieces (~23bp) of double-strand RNA formed from this breakdown would be expected to target and silence *APY1*, and our results indicate that this happens.

We developed three lines of transgenic plants harboring the RNAi construct and designed a gene-specific probe that could be used to assess transcript levels of *APY1* in them by RNA gel blot analysis (Fig. 3.2 A). After confirming the induction of the RNAi construct by estradiol in *apy2* mutants significantly depressed the expression of *APY1* (Fig. 3.2 B), we found that induction also significantly reduced the growth of all three lines, both at the seedling and flowering stages of growth (Fig. 3.2 C, D).

Although all three lines had suppressed growth, the level of growth suppression did not correlate with the level of message reduction.

In dark-grown seedlings, the most rapidly growing tissue is the hypocotyl. Shortened hypocotyl length was found in 3.5-d-old etiolated seedlings of all three RNAi lines after they had germinated and grown the entire time in a medium containing estradiol. The length of hypocotyls in *apy2* mutant plants was about 15% shorter than in the wild-type strain, and the average length of etiolated hypocotyls in RNAi lines was about 30% shorter than of wild-type control (Fig. 3.3 A). All three RNAi lines and *apy2* mutant plants had significantly shorter hypocotyl lengths compared to wild-type seedlings ($p < 0.01$) (Fig. 3.3 A).

In light-grown seedlings the most rapidly growing tissue is the primary root. Primary root growth of estradiol-treated RNAi seedlings grown in the light was analyzed from d 3 to d 6. The seedlings exhibited a significantly reduced rate of root elongation in all three lines (Fig. 3.3 B), resulting in significantly shorter roots by d 6 (Fig. 3.3 B).

In order to test if estradiol itself can inhibit root growth, the difference of root length in estradiol-treated and non-treated seedlings, both *apy2* and wild-type plants, was measured. Data showed estradiol had no effect on *apy2* mutant plants, which were used as the background plants of the RNAi lines. Although estradiol did slightly reduce the growth of wild-type roots, it reduced the root growth of the three RNAi lines to a much greater extent than did the wild-type and *apy2* mutants (Fig. 3.3 C). This demonstrated that the shorter root length of RNAi lines was not due to applying estradiol. Wild-type adult plants treated with estradiol were indistinguishable from RNAi plants that were not treated with estradiol (data not shown).

Lines suppressed in apyrase expression have fewer lateral roots, more adventitious roots

Beyond decreased growth, the most notable developmental effects of suppressing the expression of both *APY1* and *APY2* were decreased formation of lateral roots and increased formation of adventitious roots. Fourteen-d-old light grown seedlings were used in this study. The average lateral roots of *apy2* mutant were 5.8 per seedling, which is not different from wild type plants in this characteristic. However, all three RNAi lines showed reduced lateral root formation. In the lateral root measurements (Fig 3.4 A), the *apy2* mutant used as the control was not different from wild type plants in this characteristic. The same seedlings were used to count the numbers of adventitious roots initiated from the root and shoot junction. In controls more than 85% percent of seedlings have no adventitious root and less than 15% only produce one. More adventitious root formation was observed in all three RNAi mutant lines (Fig 3.4 B).

RNAi suppressed mutants have reduced elongation zones

In all three of the lines with RNAi induced suppression of apyrases, greatly reduced primary root length was observed. It is possible that this diminished growth is due to defects in a particular region of the roots. In an Arabidopsis primary root there are four main regions: root cap, meristematic zone, elongation zone, and zone of maturation. To determine whether these zones are affected in RNAi lines, scanning EM was performed to examine the structures of roots in the suppressed apyrases lines. In mutants, a high frequency of differentiated root hairs, which is a mark of the maturation

zone, was observed extending all the way from the junction of root and shoot to the root tip. The estradiol treated RNAi mutants showed a lack of a well-defined meristematic zone, a greatly reduced zone of elongation, and larger diameter root tip than wild type seedlings (Fig. 3.5). The shoot of RNAi lines was also examined and no difference was observed compared to wild type (data not shown). This corresponded with the previous promoter-GUS results that showed no expression of apyrase in light grown hypocotyls (Wu et al. 2007). These data suggest that the suppression of *APY1* and *APY2* has its primary effect on cell elongation rather than on differentiation in primary roots.

Endogenous auxin distribution was disrupted in RNAi mutant lines

The fact that high levels of ATP in the growth medium could disrupt normal auxin transport in wild type roots was previously demonstrated in Dr. Roux's lab (Tang et al. 2003). This was confirmed by the accumulation of GUS, which was driven by an auxin response promoter DR5, in root tips (Tang et al. 2003). To investigate whether the reduced root growth was caused by altered auxin distribution, an indirect test of the effects of suppressing *APY1* and *APY2* expression on auxin transport was carried out using DR5 : GFP. The transgenic DR5 : GFP plants were crossed with RNAi mutant lines to introduce DR5 : GFP into the RNAi mutants. Seedlings that exhibited reduced root growth on medium containing estradiol were selected and visualized by confocal microscopy. The endogenous auxin distribution was evaluated. The results showed that the typical "fountain" pattern of DR5 : GFP distribution in wild type primary roots was severely disrupted in estradiol-induced RNAi mutants, resulting in much more of the GFP signal in root cortex cells and in cells associated with the distal root vasculature

(Fig. 3.6). These results suggest that the suppression of apyrases could resemble the effects of treating wild type seedlings with high concentrations of ATP, and that one mode by which apyrase suppression can inhibit root growth is by disrupting the normal pattern of auxin transport.

DISCUSSION

In a previous publication Steinebrunner et al. (2003) demonstrated the essential role of two Arabidopsis apyrases in pollen germination by showing that the absence of these apyrases in pollen caused aborted germination. Pollen tubes, stigma, etiolated hypocotyls, and root tips are regions involved in rapid growth and active secretion. The abundant expression of apyrase was observed in all these fast growth areas. Promoter:GUS results showed that GUS staining disappears in light grown hypocotyls, where growth is suppressed, but there is more extensive activity of GUS in etiolated seedlings, in which the hypocotyl elongates at a remarkable speed (Wu et al. 2007).

Based on the expression pattern of apyrases, we predicted that apyrases might play a central role in growth control. Strong support for this hypothesis comes from the observation that the transgenic plants overexpressing *APY1* tagged by a six-copied myc peptide showed increased hypocotyl growth in darkness, and the level of increased growth was correlated with the expression of this APY1-myc protein. *APY1* single knockout plants and *APY2* single knockout plants were examined under the same conditions as overexpression lines. The reduced etiolated hypocotyl growth was observed in both *apy1* mutant plants and *apy2* mutant plants. This suggests that there is

a close relationship between apyrases and growth control. The idea was further supported by the RNAi mutant lines that suppress *APY1* in an *apy2* mutant background by the estradiol-inducible expression of double-stranded (ds) *APY1* RNA (dsRNA). Consistent with the previous results, the suppression of apyrases by RNAi showed reduced hypocotyl length in the dark. The RNAi mutant lines also have a dwarf phenotype with drastically reduced root and shoot growth.

The GUS expression of apyrase was found to be particularly strong in the primary root tip and the zone of elongation between day one and day five. These localization studies indicate that the function of apyrases might relate more to the growth of the primary root than the shoot. Based on the GUS results, the examination of both root and shoot growth was carried out in RNAi mutant lines. In light-grown seedlings of RNAi-suppressed plants, the growth inhibition is more evident in roots, which grow more rapidly in light-grown seedlings, than in hypocotyls. The estradiol treatment that induced the expression of double stranded *APY1* clearly suppressed normal root growth in those mutants.

The transcript levels of all three RNAi lines were evaluated by Northern blot and all three lines showed decreased levels *APY1* mRNA. The levels of decreased transcript of *APY1* were not correlated with the levels of the phenotype induced by RNAi. Previous studies have reported the lack of association between abnormal phenotype and the decrease in transcript levels (Acosta-García and Vielle-Calzada 2004, Kerschen et al. 2004). The effects of estradiol on the growth of wild type seedlings were also evaluated, and the results showed that no significant growth difference between estradiol-treated and non-treated seedlings was found. The dwarf growth of multiple lines of

plants suppressed in apyrase expression by dsRNA induction underscores a key role for apyrase in growth. The combination of the expression data and the suppression results strongly support the conclusion that apyrase expression is critical for normal, full cell expansion in Arabidopsis.

Based on the findings that the greatly reduced growth of the primary root was observed in RNAi mutant lines, we focused on the study of the function of apyrase on root development. Estradiol-treated RNAi mutants roots were examined by scanning EM. The results revealed a number of conspicuous phenotypes caused by the suppression of apyrase were found in the primary root of RNAi mutant plants, including cellular abnormalities, the missing of a well-defined meristematic zone and the highly compacted zone of elongation. An earlier report (Tang et al., 2003) showed that applied ATP increased the sensitivity of roots to exogenous auxin. Moreover, this report found that the application of 2 mM ATP in growth medium resulted in the build-up of large amounts of auxin in the root tip (Tang et al. 2003).

The effects of growth caused by auxin could be due to too little or too much auxin in the responding tissues. To investigate the possibility whether the reduced growth in the RNAi mutant plants was the consequence of altered auxin, two approaches were used in this study. If the decreased growth was caused by a lack of auxin, the exogenous application of auxin should be able to partially or fully restore the normal growth in RNAi mutant seedlings. To test this possibility, RNAi mutant seeds were germinated on the medium containing different concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D), which is a synthetic auxin. However, the results showed this treatment did not rescue the phenotype (data not shown). The expression of auxin-responsive DR5:GFP

gene was highly induced in RNAi mutant lines, suggesting an abnormal accumulation of auxin in root regions of the mutant lines.

Auxin also plays an essential role on lateral root formation. Plants treated with NPA, which is an auxin transport inhibitor, showed arrested lateral roots initiation (Casimiro et al. 2001). The formation of lateral roots was also inhibited in RNAi mutant lines. Whether this decreased lateral root initiation is due to the impaired root development or caused by the increased level of eATP is still not clear. Increased number of adventitious root formation was found in all three RNAi mutant lines, implying the effects of auxin accumulation (Blakesley 1994).

The previous studies of apyrase in animals indicated the function of this enzyme is to control the level of ATP in extracellular space and thus regulate the level of activation of purinoceptors. Potentially these two Arabidopsis apyrases could play a role as ectoapyrases. Protein structure analysis predicted that both apyrases have signal peptides. During *in vitro* Arabidopsis pollen germination remarkable amounts of apyrase activity are released into the pollen germination medium, as determined by apyrase activity assays (Wu et al. 2007). The localization and pollen tube elongation data showed early in chapter two also suggested that apyrases might be anchored on the plasma membrane and function as ectoapyrases. During root development, the mechanism through which the increased level of extracellular ATP affects the auxin distribution is not clear. One possibility is that high concentrations of extracellular ATP could inhibit the expression of multidrug resistance gene (*MDR1*) in Arabidopsis (Thomas et al. 2000). The MDR1 protein that is functional as an auxin facilitator is important in regulating the auxin transport in both shoots and roots (Noh et al. 2001,

Lewis et al. 2007). Decreased *MDR1* expression may impair auxin transport and cause the accumulation of auxin in RNAi mutant lines. Taken together, the data suggest that the function of two Arabidopsis apyrases is closely correlated with growth, possibly through the regulation of auxin transport. Further study of gene expression in RNAi mutant lines may help us to dissect the role of apyrase in root development.

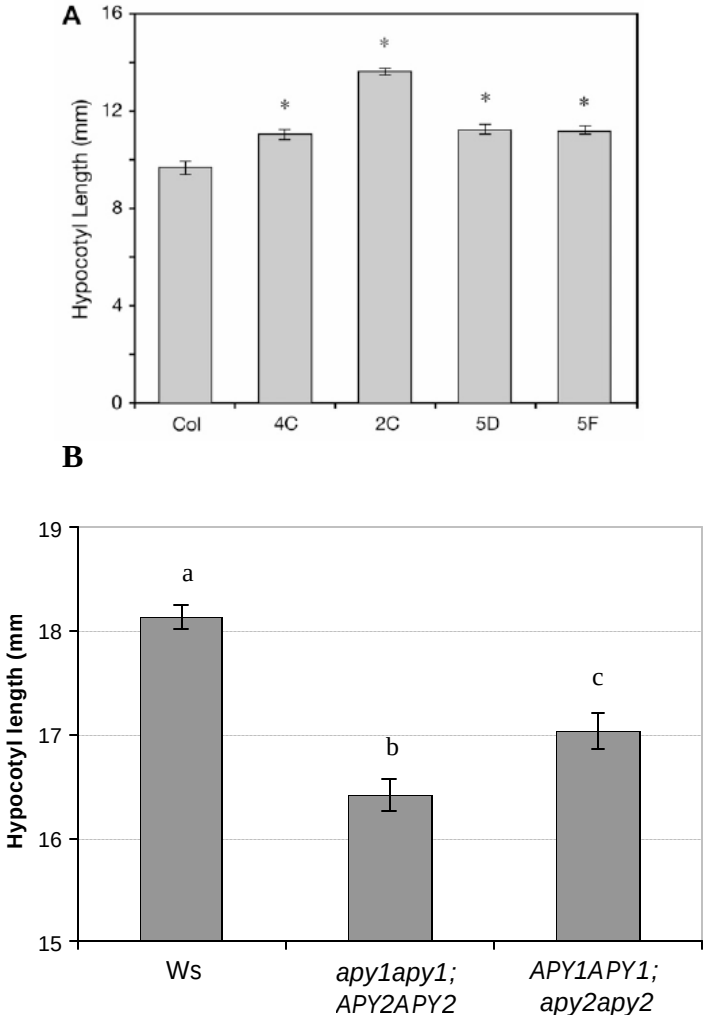

Figure 3.1 Constitutive expression of an apyrase enhances growth and single KO apyrase inhibits growth.

Enhancement of hypocotyl growth in 3.5-d-old *35S:APY1* plants overexpressing *APY1* (A). Hypocotyl length was measured in 3.5-d-old seedlings of four different lines (4C, 2C, 5D, and 5F) of *35S:APY1* plants and in Columbia ecotype wild-type plants (Col). The mean values marked with an asterisk are significantly different from mean wild-type hypocotyl lengths as determined by Student's *t* test ($P < 0.01$). Decreased hypocotyl growth in 3.5-d-old apyrases single KO mutants (B). Different letters above the bars indicate mean values that are significantly different from one another ($P < 0.01$, Student's *t* test). (This figure was published in Wu et al. 2007.)

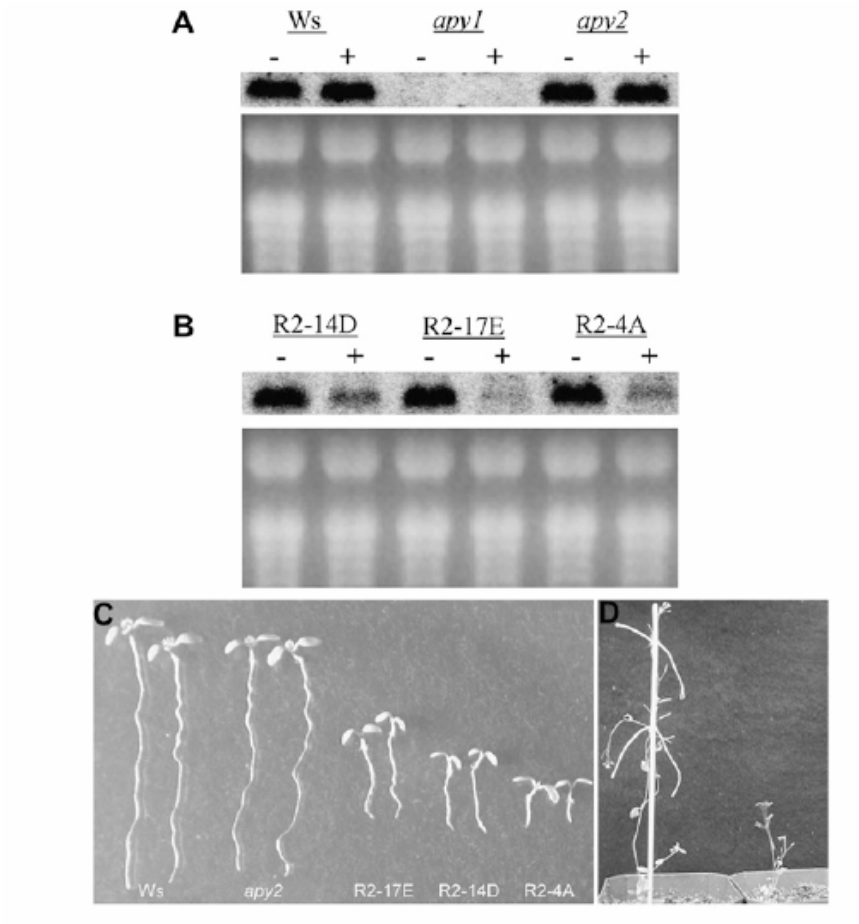

Figure 3.2 Induction of an RNAi construct targeting *APY1* in *apy2* plants reduces *APY1* transcript abundance and suppresses growth in light.

The level of *APY1* transcripts in hypocotyls of wild-type (Ws ecotype), *apy1*, and *apy2* mutants as determined by RNA gel-blot analysis (A). In both A and B, seedlings were either treated with 4 μM estradiol (+) or not treated with estradiol (–). The membrane was hybridized with a 330-bp *APY1*-specific probe containing the –350- to –20-bp upstream region of the *APY1* start codon. Abundance of *APY1* transcripts in three lines of *apy2* mutants suppressed in *APY1* by RNAi (B). Dwarf growth of *apy2* seedlings suppressed in *APY1* by RNAi (three R2 lines) compared to wild-type (Ws) and *apy2* seedlings after 7-d growth (C). Adult *apy2* mutants suppressed in *APY1* by RNAi (right) or not suppressed (left) (D). (This figure was published in Wu et al. 2007.)

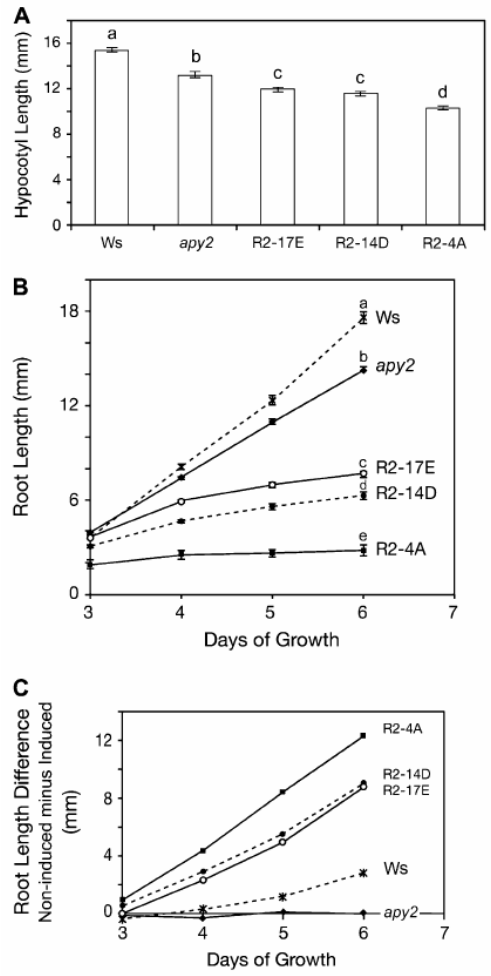

Figure 3.3 Suppression of *APY1* expression in *apy2* plants suppresses growth of hypocotyls and roots.

Suppression of growth in etiolated hypocotyls of *apy2* plants expressing an RNAi construct for *APY1* (A). The hypocotyl length was measured in 3.5-d-old seedlings that had been grown the entire time in estradiol to silence *APY1*. Different letters above the bars indicate mean values that are significantly different from one another ($P < 0.01$; $n > 20$, Student's *t* test). Suppression of root growth in light-grown *apy2* plants expressing an RNAi construct for *APY1* (B). Growth was assayed from days 3 to 6 of RNAi lines (R2-17E, R2-14D, and R2-4A), wild-type (Ws), and *apy2* mutant lines. Different letters above the bars indicate mean values that are significantly different from one another ($P < 0.01$; $n > 20$, Student's *t* test). Difference in root length between estradiol-treated and not treated RNAi lines, wild type (Ws), and *apy2* from days 3 to 6 (C). (This figure was published in Wu et al. 2007.)

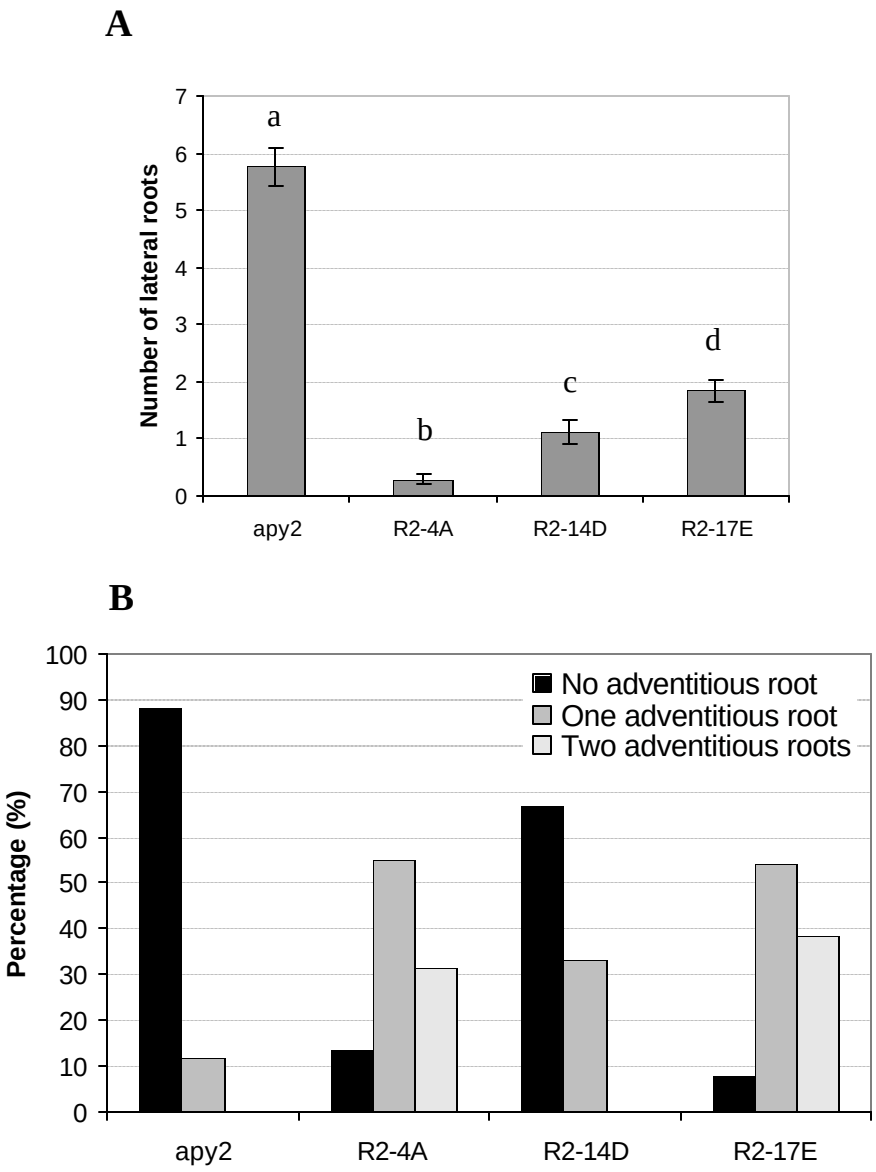

Figure 3.4 Lateral root and adventitious root measurements in RNAi mutant lines.

Numbers of lateral roots were measured in 14-d-old light-grown seedlings in RNAi mutants (A). Different letters above the bars indicate mean values that are significantly different from one another ($P < 0.05$; $n = 28$, Student's t test). Percentage of adventitious root formation in RNAi mutants compared with *apy2* (B). Error bars are ± SD. (This figure was published in Wu et al. 2007.)

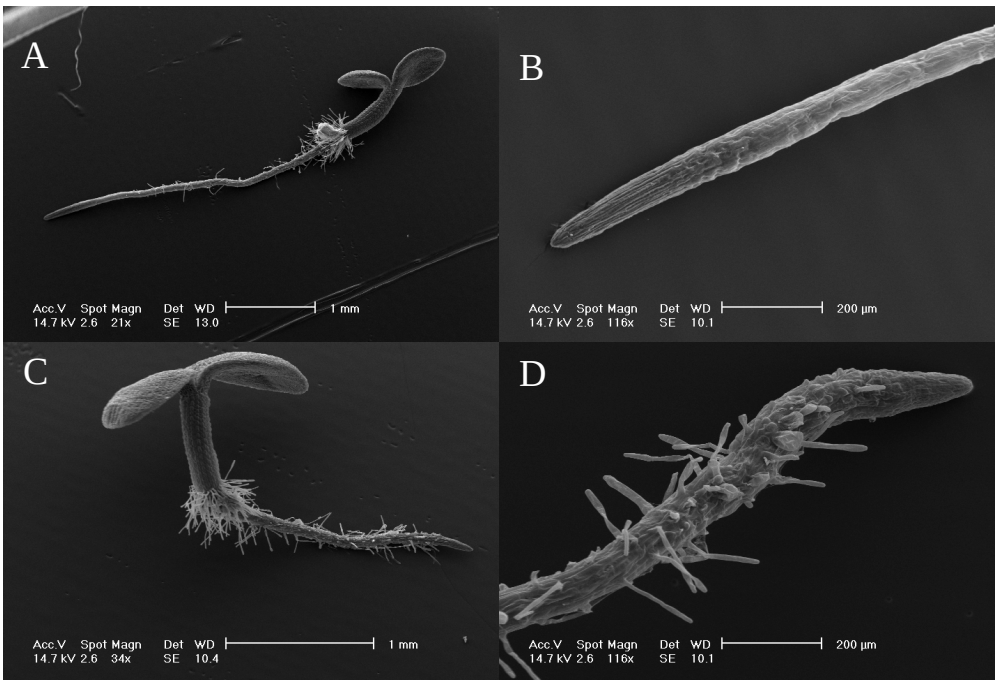

Figure 3.5 Scanning electron microscopy image of RNAi mutant.

Six-d-old **w**ild-type whole seedlings (A) and enlarged apical zone of root (B). Six-d-old R2-4A whole seedlings (C) and enlarged apical zone of root (D).

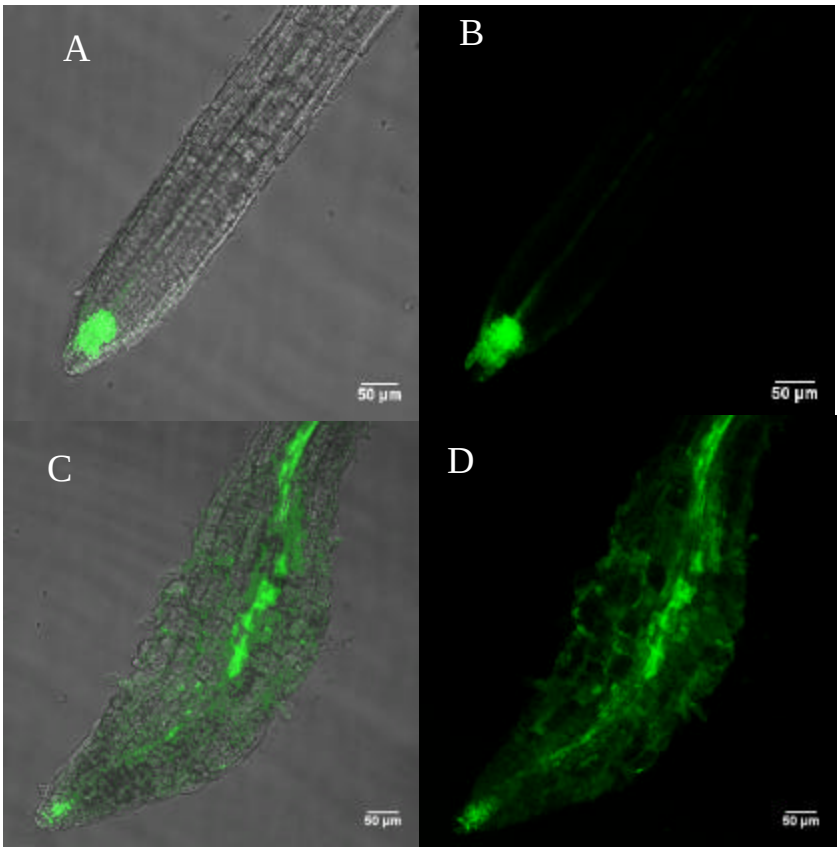

Figure 3.6 Confocal image of endogenous auxin distribution in RNAi mutant lines.

Six-d-old R2-4A shows altered DR5:GFP expression (C, D) compared with wild-type control (A, B).

CHAPTER 4: CONCLUSION

The existence of extracellular ATP (eATP) as a signal molecule was first observed in animals in 1959 (Holton, 1959) and has been well studied since then. To maintain the normal function and physiological processes of many different cell types, the level of eATP needs to be tightly regulated in the extracellular milieu. The metabolism of eATP is controlled by enzymes that exist in the extracellular matrix, and among the most important of these are ectoapyrases.

In Arabidopsis, two apyrases were previously characterized as having four apyrase conserved regions and expected nucleoside triphosphate diphosphohydrolase activities. These two apyrases are highly similar at the amino acid level and both carry signal peptides as judged by protein structure analysis (Steinebrunner et al. 2000). Genetic approaches were used to probe the functions of these two apyrases. The analysis of apyrase single knockout null mutants revealed that neither *apy1* nor *apy2* showed any morphological difference from wild-type plants (Steinebrunner et al. 2003). In addition to this, the mRNA level of one apyrase did not increase in the background of knocking out the other apyrase. This indicated that *APY1* and *APY2* are genetically redundant and have overlapping functions. On the other hand, we found that the double homozygous mutant was not viable. This suggests that in Arabidopsis, at least one apyrase should be maintained for normal growth and development.

The lethality of the *apy1apy2* double knockout (DKO) could be caused by a defect in fertility, embryo-lethality or loss of viability in an early developmental stage

after germination. Nevertheless, no reduced viability of seedlings or defects in embryogenesis was detected in heterozygous mutants that produce 50% DKO mutant progenies. Therefore, the disruption of apyrase resulted in sterility due to failed germination of the male gametophyte. *In vitro* studies of pollen germination revealed that the defect in pollen germination resulted in the infertility of the DKO. The examination of viability showed that the apyrase-deficient pollen is viable but fails to germinate. This was further confirmed by a tetrad study using the *qrt* mutant. Moreover, wild-type pollen germination was inhibited by external application of ATP in the germination medium (Steinebrunner et al. 2003).

The coincidence that high concentrations of ATP could inhibit pollen germination and knockouts for both apyrases could completely block pollen germination indicated that the possible function of apyrase in pollen germination is to maintain an optimal eATP concentration. Apyrase activity was not only necessary in pollen germination but also in pollen tube growth, so these enzymes play an important role in both events.

The pollen tube elongation assay indicated that both anti-apyrase antibody and chemical apyrase inhibitors have an inhibitory effect on *in vitro* pollen tube growth. Since the antibody cannot cross the pollen tube plasma membrane, these data in combination with the results of transient expression assays in onion skin suggest that apyrases might function on the plasma membrane. Alternatively, since reduced apyrase activity was detected in pollen germination medium when treated by apyrase antibody and apyrase inhibitors (Wu et al. 2007), the secretion of apyrases to the outside of the cell could be necessary to regulate the [eATP] in pollen tube elongation.

Song et al. (2006) reported that the expression of apyrases could be trigged by high levels of ATP released from wound sites in Arabidopsis leaves, and overexpressing APY2 in Arabidopsis results in the hyposensitivity of leaves to wound-released ATP (Song et al. 2006). Significant accumulations of eATP were also found in pollen germination medium in which the apyrase antibody was applied (Wu et al. 2007). All of this evidence indicates that at least some fraction of APY1 and APY2, like some apyrases in animals, function as ectoapyrases, thereby regulating eATP.

Exactly which processes in pollen germination involve eATP still needs further study. Recently, Prado et al. (2004) reported that nitric oxide (NO) is involved in regulating pollen tube growth and guidance. The NO signaling pathway, which can trigger plant defense responses and regulate plant development (Lamattina et al. 2003, He et al. 2004), is activated in pollen germination and pollen tube elongation in response to elevated eATP (S. Reichler et al. unpublished data). Thus the inhibitory effects caused by eATP in pollen germination and tube growth may be mediated through increased NO production and the downstream signaling changes triggered by NO.

Because of the difficulty in generating DKO plants, RNAi mediated disruption of APY1 activity in the *apy2* knockout background was used as a substitute to suppress the expression of apyrases in seedlings and mature plants. A BLAST search of the whole genome of Arabidopsis revealed that, except for *APY1* and *APY2*, there are no other genes that have sequences similar to that used in the RNAi construct, including other apyrases, so it is unlikely that the mutant phenotypes can be attributed to suppression of other apyrase genes in RNAi-silenced mutants. Consistent with the pollen tube growth data, the RNAi lines showed reduced length of roots and etiolated hypocotyls, and a dwarf

phenotype. The study of root structure through SEM revealed reductions in the elongation zone and a lack of a well-defined meristematic zone in RNAi mutant seedlings. Pollen tubes, etiolated hypocotyls, and the zone of elongation in primary roots are areas of active growth and secretion. These phenotypes combined with promoter-GUS results suggest that Arabidopsis apyrases are a key regulator in plant growth.

Previously results showed that low levels of eATP could enhance etiolated hypocotyl growth and pollen tube elongation and dramatically inhibit these processes when the concentration of eATP rises above a certain level (Roux et al. 2006, S. Reichler et al. unpublished data). Therefore the existence of an optimal level of ATP may be essential for normal biological processes and development in plants. If the rate of eATP accumulation and depletion is controlled by ectoapyrases, the disruption of apyrase function will result in accumulation of eATP and thus further inhibition of growth. This is consistent with the RNAi mutant results, which showed reduced growth when the expression of apyrases was suppressed, and the data obtained from overexpression APY1 lines that exhibited the enhanced hypocotyl growth in etiolated seedlings. Additionally, decreased growth was observed after the application of high concentrations of potato (*Solanum tuberosum*) apyrase, which can efficiently degrade eATP. Since the depletion of eATP can initiate cell death (Kim et al. 2006, Chivasa et al. 2005), it is essential to maintain an optimal level of eATP for a normal plant growth and this is achieved, at least in part, by apyrases. Coincidently, the expression of apyrases is associated with rapidly-growing and actively-secreting areas, where eATP might be released, such as pollen tips, etiolated hypocotyls, and light-grown roots, especially root tips.

How could an increase in [eATP] be linked to a decrease in growth? A possible way that eATP regulates growth is through auxin, which is a crucial hormone in controlling cellular process, such as growth, gravitropism, elongation, and differentiation in plants. That high levels of auxin inhibit growth has been demonstrated repeatedly in the literature, including recently by Hu et al. (2005), who showed that the asymmetric accumulation of auxin on the lower side of roots induces nitric oxide production thereby leading to asymmetric growth inhibition and gravitropic bending. The study of auxin distribution in RNAi mutant lines showed that high levels of auxin were accumulated in the root vascular bundle and root tip. This is consistent with the idea that an increase in [eATP] can block auxin transport and promote auxin accumulation in apical zone of wild-type roots (Tang et al. 2003).

If eATP regulates growth through auxin, what is the downstream component that connects eATP to auxin transport? One candidate is MDR1, which is an auxin efflux facilitator protein (Lin and Wang 2005, Geisler and Murphy 2006). A speculation based on this connection would be that the inhibitory effect on MDR1 by elevated levels of eATP would lower MDR1 transport activity and thus cause auxin to accumulate up to levels that diminish growth. To the extent that high eATP can lead to the accumulation of growth-inhibitory levels of auxin (and/or of NO), preservation of lower eATP through apyrase activity at growth points may be needed to maintain growth.

Thus, in the future, it will be interesting to test whether the suppression of apyrases can be overcame by overexpression of MDR1. Moreover, tests of whether the addition of ATP-?-S can block the increased etiolated hypocotyl growth caused by overexpression of apyrases could provide more evidence of the importance of eATP in

growth control in Arabidopsis. Such additional studies would help us to produce a better understanding of the function of apyrases in eATP signaling in plants.

On the other hand, the production of superoxide is generally linked to cell elongation and growth promotion (Schopfer and Liszkay 2006). An earlier publication by Song et al. (2006) provided strong support for induction of superoxide production by eATP (Song et al. 2006). This suggests that control of growth by apyrase could occur through the regulation of superoxide production. If low [eATP] is necessary for keeping normal levels of superoxide to maintain plant growth, the increased [eATP] by the disruption of apyrase function could lead to the overproduction of superoxide, resulting in levels that are sub-optimal for growth, perhaps by elevating nitric oxide, which can act antagonistically to superoxide (Thomas et al. 2006).

Taken together, the data in this dissertation reveal that two closely related apyrase enzymes can regulate the [eATP] of plant cells, and their activity is closely correlated with growth and, in fact, is needed for normal growth in Arabidopsis. We propose that an optimal level of eATP in the extracellular space is essential for plant cell growth and this mechanism is controlled, in part, by the function of APY1 and APY2.

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